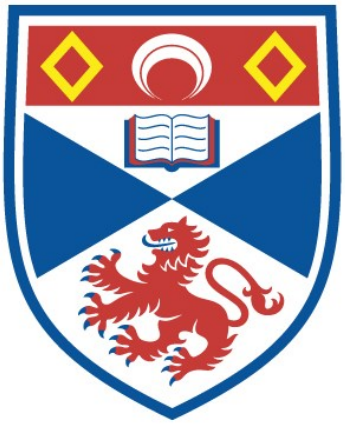
ANTIOXIDANT PROPERTIES OF ETHOXYQUIN AND SOME OF ITS OXIDATION PRODUCTS

Snorri Thorisson

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1988

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A Thesis

presented for the degree of

DOCTOR OF PHILOSOPHY

in the Faculty of Science of the

University of St Andrews

by

SNORRI THORISSON, BSc

November 1987

United College of St Salvator
and St Leonard, St Andrews

CERTIFICATE

I certify that *SNORRI THORISSON* has been engaged in research at the University of St Andrews, has fulfilled the conditions of Ordinance General No 12, and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy. He was admitted under this Ordinance in January 1984.

I certify that the following thesis is based on the results of research carried out by me, that it is my own composition, and has not previously been presented for a higher degree.

ACKNOWLEDGEMENTS

I would like to express my thanks to the following: Professor F.D. Gunstone (St Andrews) and Dr R. Hardy (Torry Research Station, Aberdeen) for their help and supervision, and Dr J.C. Walton for his help concerning the ESR spectroscopy.

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I also like to thank Nina Costa for proofreading my thesis and Catherine Finlay for the typing.

My greatest thanks go to my family and friends for their constant support and encouragement.

ABSTRACT

Ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (I)), ethoxyquin nitroxide (II), 2,6-dihydro-2,2,4-trimethyl-6-quinolone (III) and 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline (IV) were prepared and purified in order to study their antioxidant properties in fish meal and/or oil. I (also commercally available) and III were equally effective in fish meal, but III and IV were better in oil than I and II.

In AIBN initiated methyl linoleate autoxidation, the order of efficiency in terms of the stoichiometric factor (n), using BHT as a standard, was IV ≫ I > BHT ≫ II. On the other hand, III gave very little inhibition in this system.

In fish meal and oil, I was converted to III and to a 1,8'-dimer (V). The same products were formed in tert-butoxy oxidation of I. The nitroxide (II) was also formed from I but this was only seen by ESR in various organic solvents. It is likely to play some role in the antioxidant mechanism of I.

The 1,8'-dimer was isolated and found to have no antioxidant properties in oil. Another reaction product of I, 2,4-dimethyl-6-ethoxyquinoline was also prepared. It was found to show prooxidant effects in fish oil.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Lipid oxidation

Oxidative deterioration can be of great importance in products containing unsaturated centres. Unsaturated fatty acids react non-enzymically with oxygen by autoxidation and photooxygenation or they may also be oxidized in enzymic reactions. The most important oxidation reaction in oils and oil-containing products is autoxidation.

Autoxidation

Autoxidation is a radical chain-reaction involving three steps; initiation, propagation and termination.

An allylic, resonance-stabilized, radical is formed through reaction of unsaturated compounds (RH) with various initiators (I·).

Initiation $RH + I\cdot \longrightarrow R\cdot + IH$

An example is the abstraction of hydrogen from the diallylic carbon-11 in linoleate.

The linoleate radical then reacts with oxygen to produce finally a mixture of conjugated 9- and 13-diene hydroperoxides.

The initiator can be formed by direct thermal dissociation, metal catalysis or photolysis, but most often it is a product of

hydroperoxide decomposition. When hydroperoxides are present in low concentration the decomposition is monomolecular, often catalysed by metals.

$$ROOH + M^{n+} \longrightarrow RO\cdot + OH^- + M^{(n+1)+}$$

and $$ROOH + M^{(n+1)+} \longrightarrow ROO\cdot + H^+ + M^{n+}$$

At higher concentrations of hydroperoxides the decomposition mechanism becomes bimolecular.

$$2\ ROOH \longrightarrow ROO\cdot + RO\cdot + H_2O$$

The propagation, like the initiation, depends on how easily the unsaturated centre donates a hydrogen.

Propagation

$$R\cdot + O_2 \longrightarrow ROO\cdot$$

$$ROO\cdot + RH \longrightarrow ROOH + R\cdot$$

The allylic radical reacts first with oxygen, in a very fast reaction, to form a peroxy radical which then abstracts a hydrogen from another unsaturated centre in a much slower, rate determining, reaction.

In the absence of inhibitors, the termination reactions are interactions of the various radicals to form non-radical products.

Termination

$$2\ ROO\cdot \longrightarrow$$
$$ROO\cdot + R\cdot \longrightarrow$$
$$2\ R\cdot \longrightarrow$$

} Non radical products

At moderate oxygen pressures, the most important reaction is between two peroxy radicals which, for secondary peroxy radicals at room temperature, furnishes a tetroxide intermediate which yields a ketone, an alcohol and oxygen.

Secondary oxidation products are very many and include aldehydes, ketones, alcohols and higher molecular weight products.

Photooxygenation

Oxygen, normally in the more stable triplet state, can form singlet oxygen by electronic excitation from the interaction of light and a sensitizer such as chlorophyll. Electronic excitation is also believed to occur through a direct reaction between transition metals and oxygen. Singlet oxygen adds to an unsaturated carbon atom and hydroperoxide is formed. This ene reaction occurs with double bond migration.

Photooxygenation of linoleate produces a mixture of four diene hydroperoxides.

9-OOH Δ 10,12 (35%)
10-OOH Δ 8,12 (17%)
12-OOH Δ 9,13 (17%)
13-OOH Δ 9,11 (32%)

Photooxygenation is much faster than autoxidation (10^3 - 10^4) and it has been suggested that autoxidation is initiated by the

breakdown of hydroperoxides, first formed by photooxygenation (1-9).

Antioxidants

Autoxidation causes a decrease in quality. Food and feed stuffs become rancid with consequent loss in nutritional quality, and products such as rubber lose their characteristic properties.

Antioxidants are compounds that inhibit or slow down the reaction between oxygen and the unsaturated compound. Their importance has long been recognised by various industries, and recent research has shown that they are also very important in life processes.

Preventive antioxidants

Preventive antioxidants are compounds that inhibit or decrease oxidation by removing potential chain initiators.

Peroxide decomposing compounds are probably the most important preventive antioxidants. Ideally, they should decompose the hydroperoxides to form non-radical products. Various sulphur containing antioxidants fall into this category (10).

Compounds such as EDTA, polyphosphates and citric acid inactivate pro-oxidative metal ions (11).

Preventive antioxidants can also operate through their reactions with oxygen. Ascorbic acid can act as an oxygen scavenger (11) and carotene is a singlet oxygen quencher (12).

Chain-breaking antioxidants

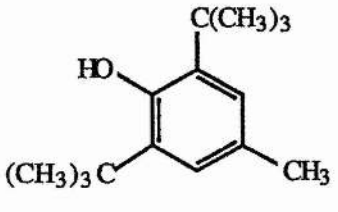
The interaction between chain-breaking antioxidants and the radicals produced by autoxidation are oxidation-reduction reactions.

Electron donating chain-breaking antioxidants are oxidized in the reaction, but electron accepting chain-breaking antioxidants are reduced.

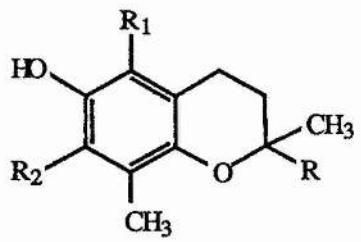
Probably the most important antioxidants are the electron donating chain-breaking antioxidants. They inhibit the progress of an autoxidation chain by donating a hydrogen atom to the peroxy radical to form a hydroperoxide, whilst the antioxidant itself produces a stable free-radical which does not initiate nor propagate further oxidation.

$$ROO\cdot + AH \longrightarrow ROOH + A\cdot$$

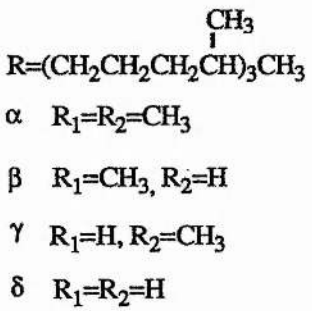
Electron donating chain-breaking antioxidants are usually aromatic compounds with a hydroxy or a secondary amine group. The most commonly used synthetic phenolic compounds are BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), TBHQ (tertiary-butylhydroquinone) and PG (propyl gallate) (11). Compounds, produced *in vivo*, that act as electron donators through hydroxy groups include ascorbic acid (13), tocopherols (14-16) and various phenols such as rosmarydiphenol isolated from rosemary leaves (17).



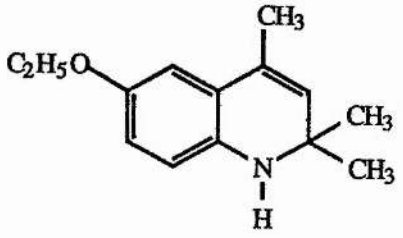
BHT



Tocopherol



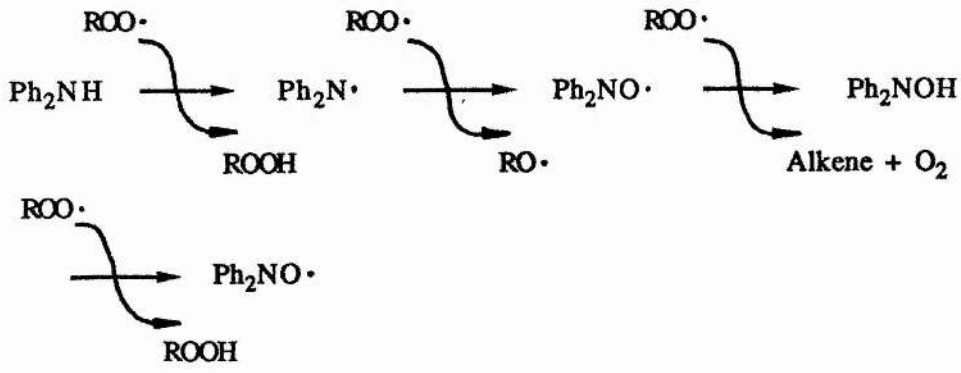
Secondary amines also react as electron donating chain-breaking antioxidants. Various arylamines such as N,N-diphenylamine and 1,2-dihydro-2,2,4-trimethylquinoline are used in rubber products (10). Ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) is a very important antioxidant for products such as fish meal.



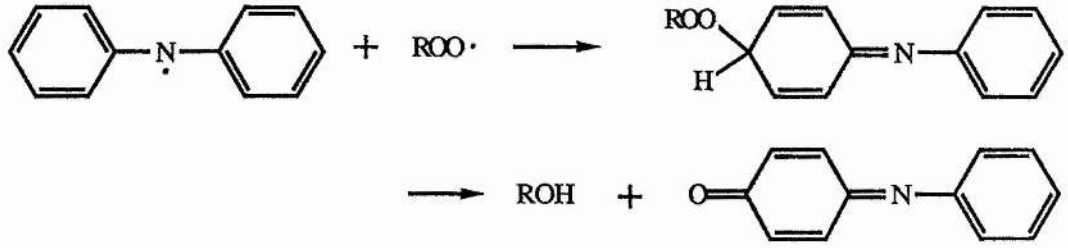
Ethoxyquin

It is known that both secondary alkyl- and arylamines produce oxidation products that are themselves antioxidants (10, 18-22) which brings us to the electron accepting chain-breaking antioxidants.

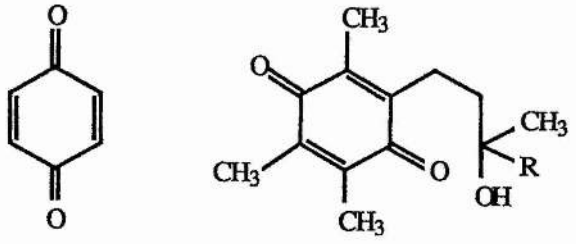
A possible scheme for the mechanism of the inhibition by a secondary aryl-amine can be as shown below (18,23).



In addition to this mechanism, a radical addition on the ring of the aromatic amine-radical is possible, producing in this case a quinone-imine (24).



Oxidation products of phenolic type antioxidants are also known to have strong antioxidant effects. Those are either resonance-stabilised radicals, formed after abstraction of hydrogen from the phenol, or various quinones eg tocopherylquinone, vitamin K, benzoquinone or rosmaryquinone.



Benzoquinone

α-Tocopheroquinone

The effects of quinones have often been related to low oxygen pressure and therefore a direct reaction with alkyl radicals, but this is not always the case (10, 16, 25).

As many of the antioxidants mentioned above are used in food

and feedstuffs, the possibility of toxcicity must be considered. An interesting way to reduce the absorption of the antioxidant is to use high molecular weight polymers (26, 27).

Ethoxyquin as an antioxidant

Ethoxyquin is mainly used in products such as fish meal and various animal feeds, to inhibit lipid autoxidation and to preserve carotene and vitamin A (28-31). It is also used as a post-harvest dip for apples to prevent scald (brown spots) which is thought to be caused by oxidation of α-farnesene (32-35), and to protect some pigments in spices (36). Although not commonly used in food produce, ethoxyquin can be absorbed from feed by animals, and subsequently consumed by humans from meat, milk or eggs (37, 38).

Amongst other things, ethoxyquin has been found to interfere with hepatic drug metabolism and to cause ultrastructural changes in hepatic cells (39). On the other hand, it has been shown to protect mice from toxic doses of pyrrolizidine alkaloids (40), and to reduce carcinogenic and toxic effects of various polycyclic hydrocarbons (41, 42).

As a secondary amine, ethoxyquin might be expected to form an N-nitrosoamine by reaction with nitrite, often present in food, under mildly acidic conditions. Many N-nitrosoamines are known to produce cancer in experimental animals (43). However, ethoxyquin was found to inhibit nitrosoamine formation in bacon and model systems (44, 45).

Autoxidation of fish meal

In the UK, fish meal is a product obtained by drying and grinding fish or fish waste to which no other matter has been added

(46). The world production of fish meal was over four million tonnes per annum in the early seventies (47), and given that fish oil production has remained virtually the same between 1975 and 1985 (48), it has probably not changed much.

The fish or fish waste is usually cooked, the oil and water removed by pressing and the solids dried to approximately 10% water content (46). The oil content can vary from 5-20% depending on the raw material and the processing.

Due to the high unsaturation of fish oil, fish meal is readily autoxidized with a consequent decrease in nutritional and energy value. Lipid autoxidation in fish meal also causes heat-production which can even lead to spontaneous combustion of stored meal (49, 50).

The pre-process history of the meal affects the rate of autoxidation. For example, if a low quality raw material is used, oxidation is likely to be faster. As has been mentioned before, various metals catalyse autoxidation. Heme and non-heme iron has been shown to catalyse autoxidation in many model and food systems (51, 52), and it is known that contact of fish flesh with the iron parts of fish processing equipment increases lipid oxidation (53, 54).

The risk of spontaneous heating in fish meal can be decreased by "curing" ie heating the meal and allowing the autoxidation to proceed before storing or transport (49, 55). However, if the nutritional and energy level is to be maintained, antioxidants will have to be used.

Ethoxyquin is the most commonly used antioxidant in fish meal and it has proved very efficient in preventing oxidation of lipids and other components, such as methionine (56).

The purpose of this investigation was to gain further understanding of the behaviour of ethoxyquin and its oxidation products as antioxidants.

CHAPTER 2

PREPARATION AND PHYSICAL PROPERTIES OF ETHOXYQUIN AND SOME OF ITS DERIVATIVES

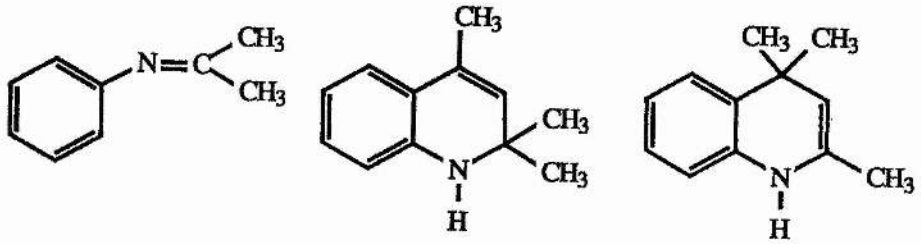
2.1 Introduction

Ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) was prepared mainly to gain experience before the preparation of the radiolabelled compound. Some of its derivatives were also prepared to examine their physical properties and effects on lipid autoxidation.

All NMR spectra are shown in Appendix I of this chapter, and the results are summarised in Tables 1 and 2 in the same appendix. Mass spectra are shown in Appendix II.

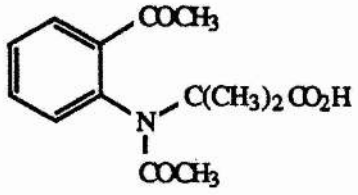
2.1.1 Ethoxyquin

The product of acid-catalysed condensation of acetone and aniline was thought by Knoevenagel (57) to be the acetone-anil (I). Craig (58) suggested that the product was 1,2-dihydro-2,2,4-trimethylquinoline (II) but could not eliminate the possibility of 1,4-dihydro-2,4,4-trimethylquinoline (III).

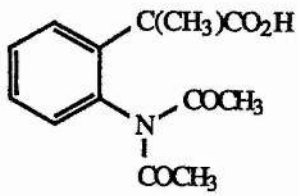


I II III

Elliott and Yates (59) pointed out that despite spectral information favouring the 1,2-dihydroquinoline, some further evidence was needed. They showed that by oxidation of the acetyl derivative of II and III with a permanganate-periodate reagent (60, 61), two possible structures IV and V, with the formula $C_{14}H_{17}NO_4$ could be obtained.



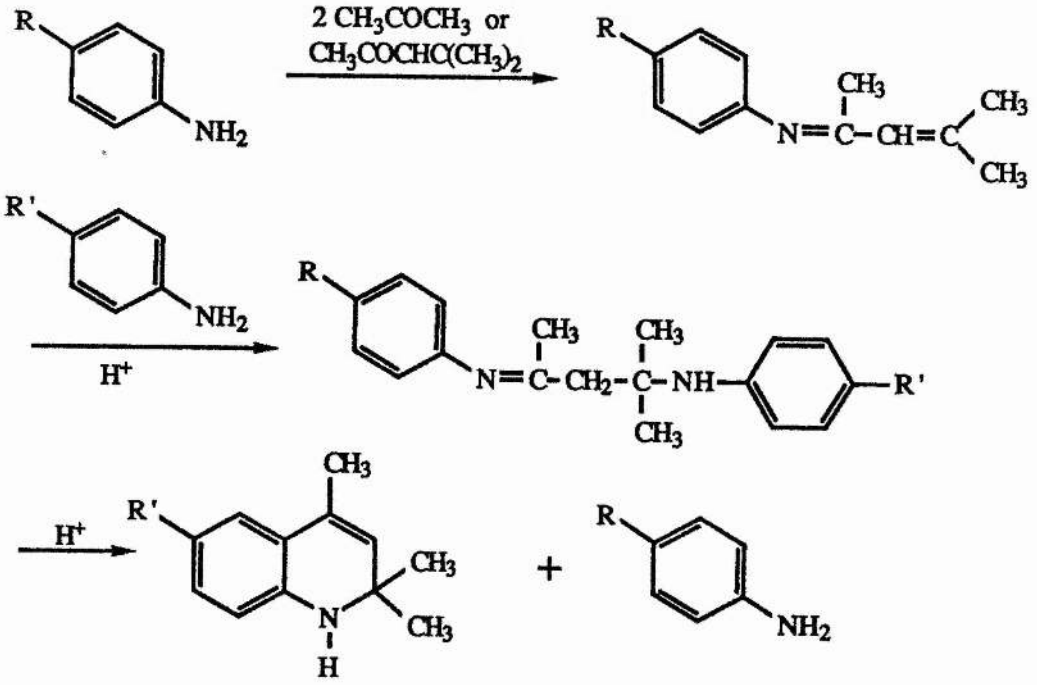
IV



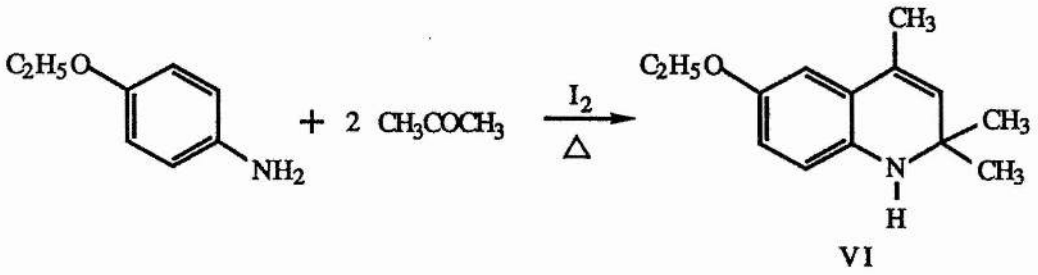
V

The product was shown to be IV, by its positive iodo form test, establishing the structure of II.

Tung (62) showed that the reaction probably proceeded by the following mechanism:



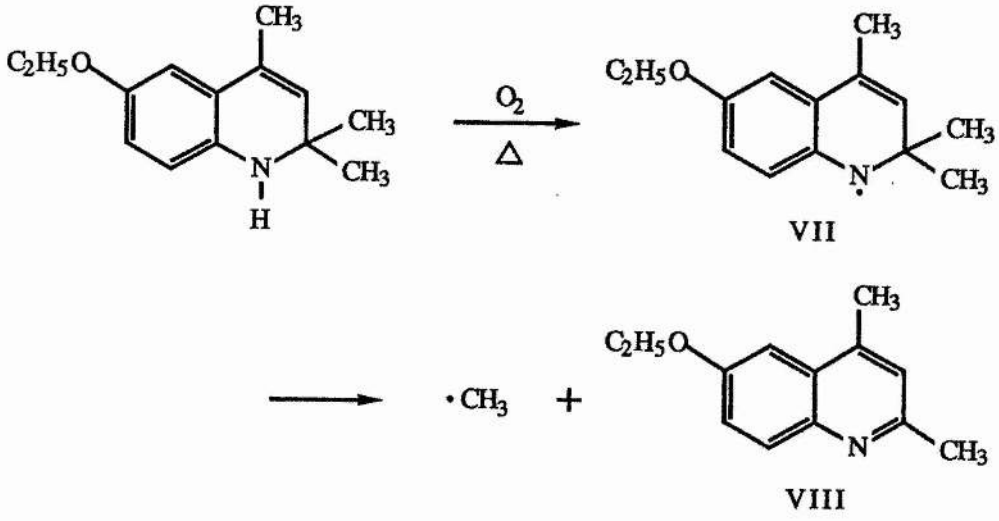
Therefore, when one mole of p-phenetidine (6-ethoxyaniline) is heated with two moles of acetone in presence of iodine, ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline) VI is formed.



VI

2.1.2 2,4-Dimethyl-6-ethoxyquinoline

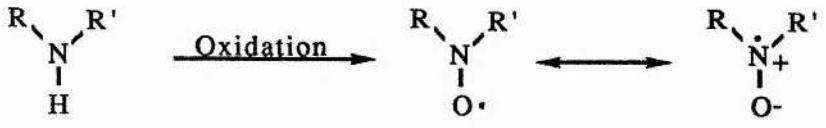
When a solution of ethoxyquin in chlorobenzene is refluxed (100 °C) in the presence of oxygen, 2,4-dimethyl-6-ethoxyquinoline (VIII) is formed almost quantitatively (63). The reaction was shown to be a radical chain-reaction, where ethoxyquin gives an unstable radical (VII) which then decomposes to form VIII and a methyl radical (64-66).



This reaction also takes place when neat ethoxyquin is heated in the presence of oxygen, and ethoxyquin decomposes in this way when injected onto a GC-column at higher temperatures.

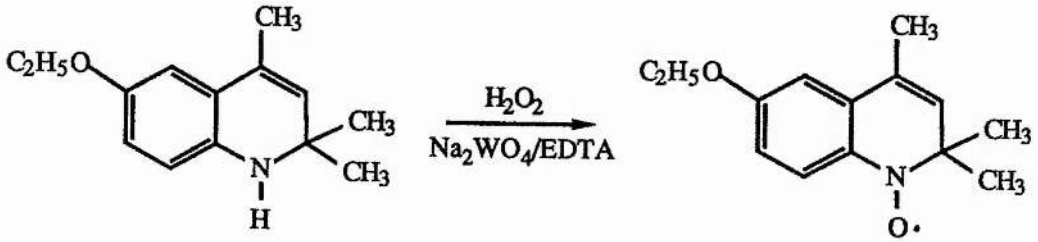
2.1.3 1,2-Dihydro-6-ethoxy-2,2,4-trimethylquinoline-nitroxide

Secondary amines can be oxidised by reagents such as hydrogen peroxide in the presence of molybdate or vanadate to form a free nitroxide radical.



Nitroxide radicals are stabilised by delocalisation of three π-electrons over the nitrogen and oxygen atoms. The energy gained by this delocalisation in two molecules is more than the energy gained by dimerisation through an oxygen-oxygen bond. Though very stable in solid state, nitroxide radicals are much less stable in solution (67, 68).

Lin and Olcott (69) claimed to have prepared and isolated ethoxyquin-nitroxide (IX) in pure form, by reaction of ethoxyquin and hydrogen peroxide in the presence of sodium tungstate and EDTA.



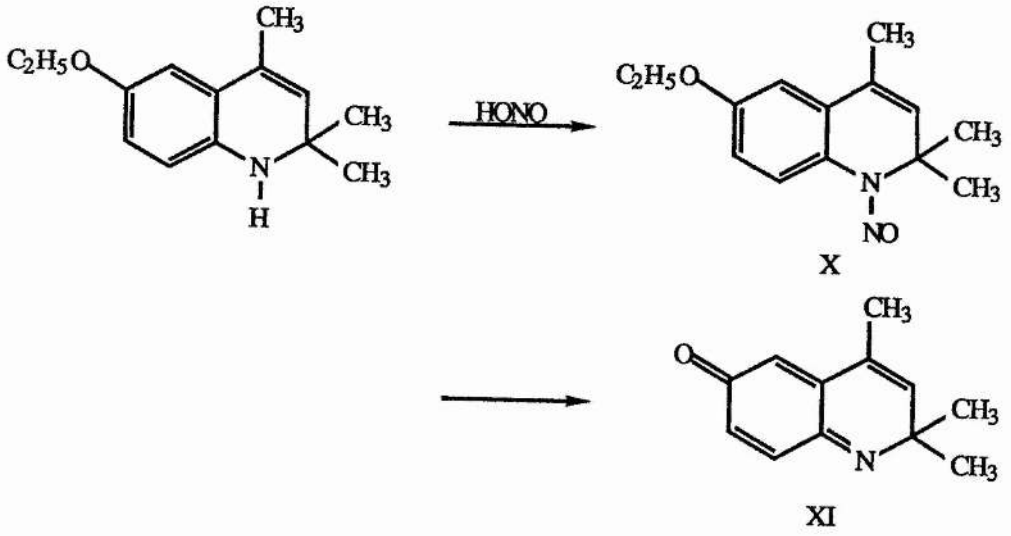
IX

They later mentioned problems of purification of the nitroxide (70).

2.1.4 2,6-Dihydro-2,2,4-trimethyl-6-quinolone

2,6-Dihydro-2,2,4-trimethyl-6-quinolone (XI) has been shown to be formed from ethoxyquin under different conditions.

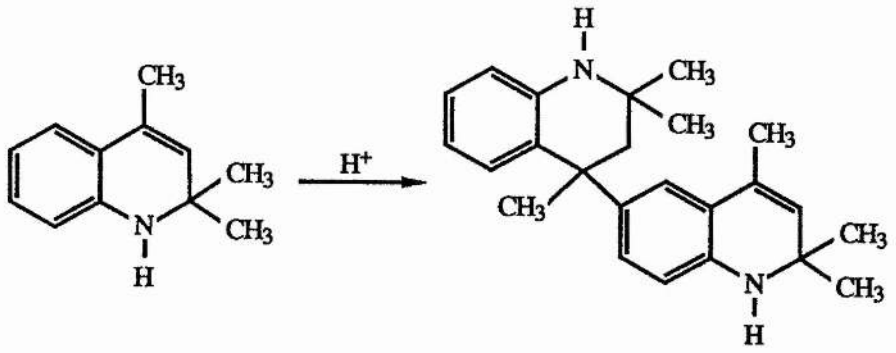
Bonnett *et al* (43), found that a reaction of ethoxyquin with nitrous acid produces primarily what is thought to be the 1-nitroso derivative (X). This compound is readily oxidised further to produce XI.



When ethoxyquin was fed to rats, XI was found in an ethyl acetate extract of urine samples, but in very small amounts and most likely as an oxidation product of 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline (71).

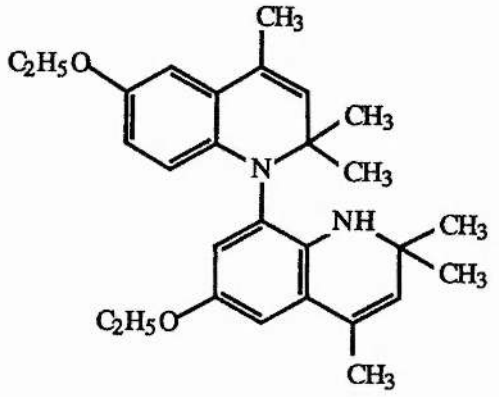
2.1.5 1,8'-Di-(1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline)

1,2-Dihydro-2,2,4-trimethylquinoline (II) forms a 4,6'-dimer (XII) when heated with a trace of acid, but ethoxyquin did not dimerise under these conditions (72).



XII

A primary reaction product of ethoxyquin in apples was a 1,8'-dimer (XIII) (33) and Kato *et al* (73), discovered the formation of the same dimer by exposing ethoxyquin to air for 4 months.

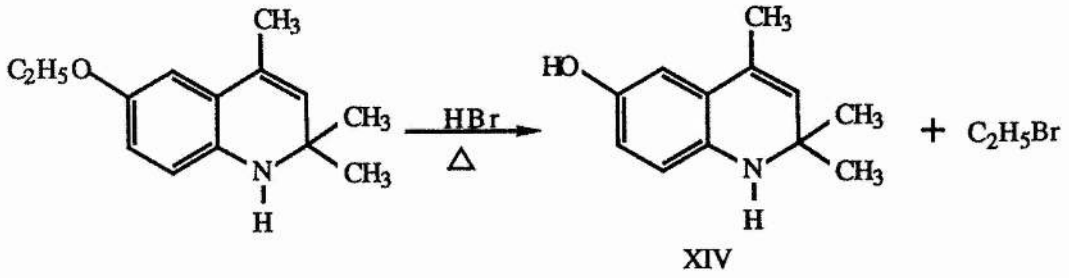


XIII

2.1.6 1,2-Dihydro-6-hydroxy-2,2,4-trimethylquinoline

Ethers can be cleaved by acids under vigorous conditions. An aryl-alkyl ether yields a phenol and an alkyl halide when refluxed with hydrogen halides, because of the lower reactivity of the aryl-oxygen bond (74).

By refluxing ethoxyquin with hydrobromic acid, 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline (XIV) is formed (75).



XIV

This compound is also formed as a metabolite of ethoxyquin in rats (71).

Results and Discussion

2.2 Preparation of labelled and unlabelled ethoxyquin

2.2.1 Preparation at different conditions

The product of the acid-catalysed condensation of acetone and p-phenetidine is 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline (ethoxyquin). Since ethoxyquin is prepared for industrial use and readily available, it was made only to provide experience for use in the preparation of the radiolabelled compound.

The reaction was carried out in a solvent to make handling of small quantities of reagents easier, and to gain better control of the reaction temperature. Of the two solvents tried, xylene proved better than toluene because of its higher boiling point (bp 137-144 ^{o}C).

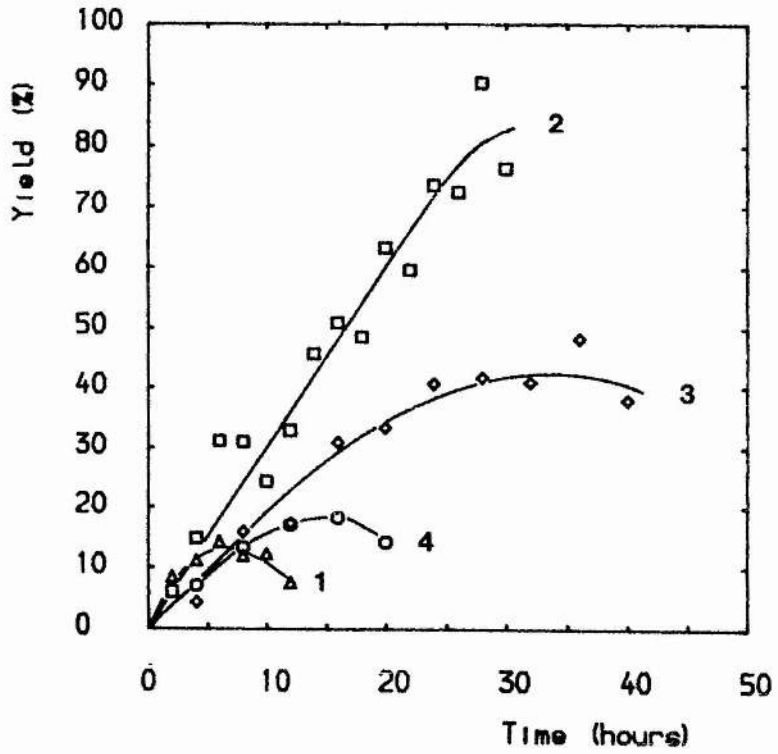
Assuming that the GC-response (FID) for a compound depends only on the ratio of carbon atom number and the molecular weight, ethoxyquin and N,N-dimethylaniline have a very similar response ($14/217.1 = 6.4 \times 10^{-1}$ and $8/121.1 = 6.6 \times 10^{-1}$). Therefore, by using N,N-dimethylaniline as a standard, the yield of ethoxyquin formation

can be calculated from the ratio of the two peaks. Because of a thermal breakdown, ethoxyquin sometimes gives two peaks, and the sum of those peak-areas is used in the calculations.

In Figure 2.2.1 the yield of ethoxyquin formed, based on acetone, is plotted against time. The second reaction gives the highest yield (90% after 28 hours), but both the GC and TLC results indicate that ethoxyquin is not completely stable under the experimental conditions. The TLC results all had a similar pattern, ethoxyquin (R_f 0.88, fluorescent, green after spraying) increasing at first and then either decreasing or staying constant. p-Phenetidine (R_f 0.39, dark blue) is seen in all the samples and at the later stages various breakdown products can be seen.

The water formed in the second reaction was measured 1.7 ml (0.09 mole) and this agrees well with a yield of approximately 90%.

Figure 2.2.1 Synthesis of ethoxyquin under various conditions, yield (%) vs time (hr).



No	Acetone (mole)	p-phenetidine (mole)	solvent
1	0.075	0.15	Xylene
2	0.1	0.1	Xylene
3	0.2	0.1	Xylene
4	0.4	0.2	none

2.2.2. Preparation of unlabelled ethoxyquin

By TLC of the ether fractions, it was seen that the first two acid extracts removed primarily unreacted p-phenetidine. After removing the ether, the last two fractions together yielded 38.8 g (26.3 and 12.5 g) of impure ethoxyquin.

Ethoxyquin came off the adsorption chromatography column between 1100 and 3000 ml, and after removal of the solvent a yellow

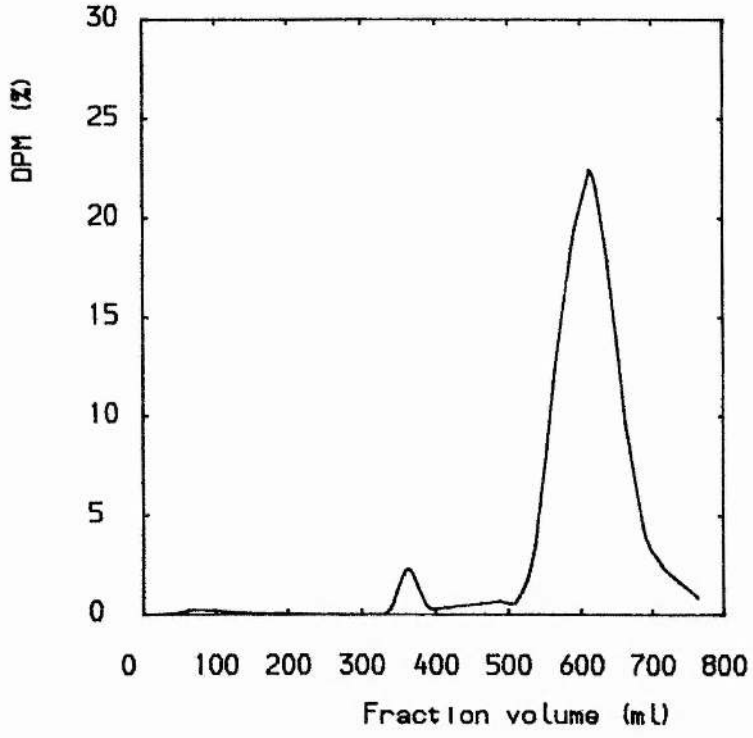
oil (15.1 g, 28% yield) was obtained. Two minor impurities were seen by TLC, one small spot (fluorescent) in front of ethoxyquin, the other (green) was just behind.

2.2.3 Preparation of ^{14}C-ethoxyquin

Before starting to heat the reaction mixture, it already showed three TLC spots; a big spot for p-phenetidine (R_f 0.14), a small unknown spot (R_f 0.43, green) and an even smaller one for ethoxyquin (R_f 0.50). The plate scan gave low counts at R_f 0.43 and 0.50, and some count was also observed at R_f 0.14 which is likely to be either an intermediate or traces of acetone carried by the p-phenetidine. The spots at R_f 0.43 and 0.50 indicate that the reaction starts immediately on mixing at room temperature. After heating, ethoxyquin gave a big spot with a high count, and a dark spot with a high count (seen without spraying) was apparent on the baseline. p-Phenetidine gave as before a big spot with very low counts.

In Figure 2.2.2 the radioactivity counts in each fraction from the chromatography column are shown as a percentage of total activity. It shows a peak, running in front of ethoxyquin, containing 3% of the total counts. Fractions 23 - 29 (550 - 725 ml) were combined to give yellow oil (0.47 g, 25.5% yield). Total count in those fractions was 1.54 x 10^8 dpm (69.9 μ Ci, 13.9% yield) which gives an activity 149.2 μ Ci/g.

Figure 2.2.2 Purification of ^{14}C-ethoxyquin by adsorption column chromatography. Radioactivity counts in each fraction (% of total) vs elution volume (ml).



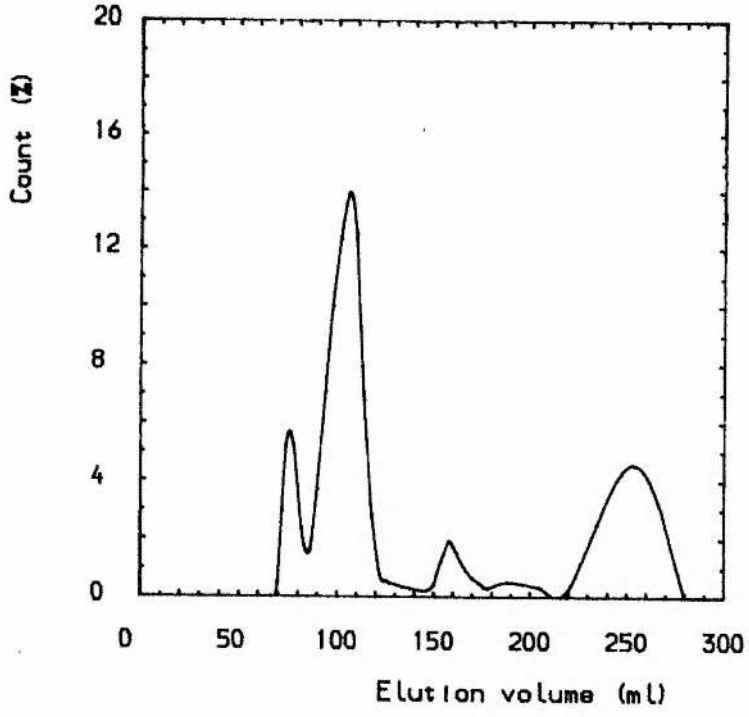
The second preparation produced a very dark oil which was purified by preparative HPLC. Table 2.2.1 shows the fractions collected from each preparative run (>99% ethoxyquin by HPLC).

Table 2.2.1 Fractions collected from preparative HPLC

Run	Fraction number	Elution volume (ml)
1	9-18	96-132
2	8-18	92-132
3	7-15	92-124
4	6-14	92-124

Figure 2.2.3 shows the counts in each fraction from the second run as a percentage of total counts against the elution volume.

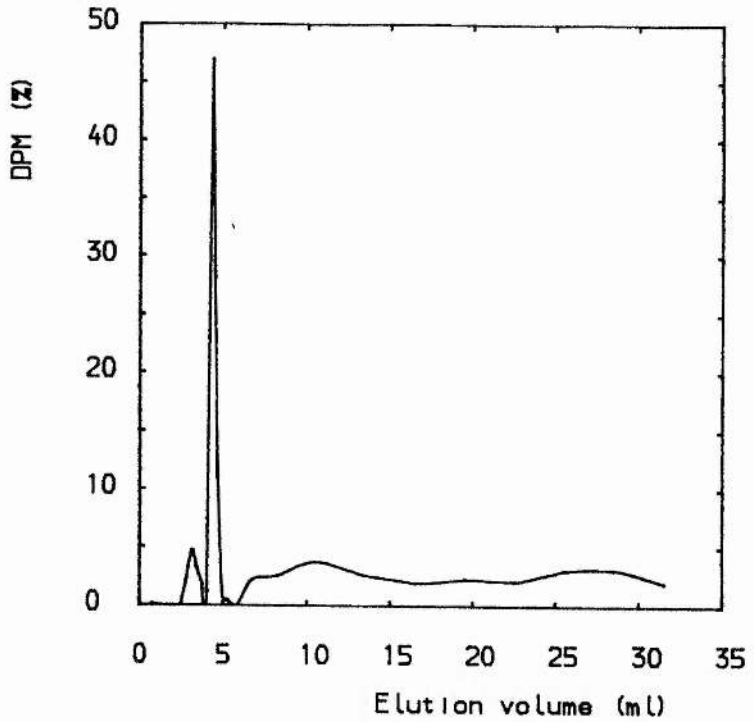
Figure 2.2.3 Purification of ^{14}C-ethoxyquin by preparative HPLC. Radioactivity counts in each fraction (% of total) vs elution volume (ml).



Due to the bigger volume of the last fractions, their peak areas are not in proportion to the count percentage. In addition to the ethoxyquin peak, three major peaks can be seen. They contain approximately 15, 7 and 5% of the total counts respectively, whereas the ethoxyquin peak contains 70%.

The same sample was injected onto an analytical column and fractions collected and radioactivity measured (Figure 2.2.4). The main peak (ethoxyquin) contains 60% of the total counts, and the first peak contains 12%.

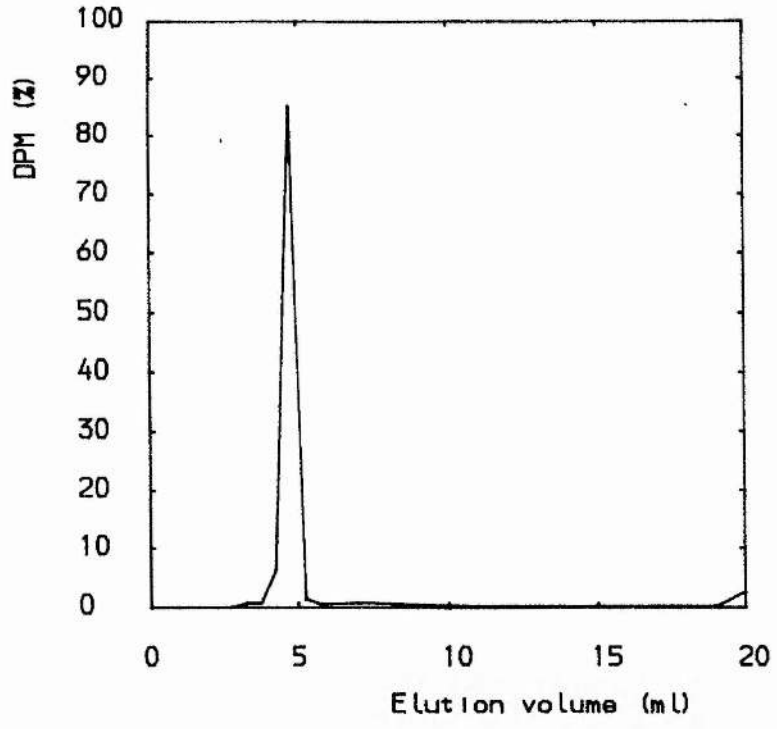
Figure 2.2.4 ^{14}C-Ethoxyquin before purification. Counts (% of total) in fractions from an analytical HPLC column vs elution volume (ml).



The combined fractions from the preparative HPLC purification of ^{14}C-ethoxyquin (0.67 g, 35.8% yield) gave a total count of 2.47 x 10^8 dpm (111.3 μ Ci, 22.2% yield) so the activity was 166.1 μ Ci/g.

The purified ^{14}C-ethoxyquin gave only one peak by HPLC. The counts in each fraction are shown in Figure 2.2.5, and apart from a very low count in the methanol fraction, only one peak is seen.

Figure 2.2.5 Purified ^{14}C-ethoxyquin by analytical HPLC. Counts in fractions (% of total) vs elution volume (ml).



2.3 Spectroscopic properties of ethoxyquin

The ^{1}H-NMR spectrum shows peaks for the ring substituents at 1.25 ppm (C(2)-$(CH_3)_2$, s, 6H), 1.98 ppm (C(4)-CH_3, d, 3H, J ~2.5 Hz), 1.37 ppm (-CH_2-$\underline{CH_3}$, t, 3H, J ~7.5 Hz) and 4.05 ppm (-CH_2-, q, 2H, J ~7.5 Hz). There are two broad peaks at 3.42 ppm (N-H, s, 1H) and 5.45 ppm (C(3)-H, d, 1H, J ~2.5 Hz) but the resolution of the aromatic region peaks is not good enough to allow definite interpretation. This spectrum agrees well with previously published results (62). By comparision to other compounds the ^{13}C-NMR spectrum has almost been interpreted fully.

In the mass spectrum, the molecular ion of m/e 217 (M^+, 36%) loses a methyl group to give an ion of m/e 202 (M^+-15, 100%), or the

ethoxy group to give an ion of m/e 188 (M^+-29, 11%). The m/e 174 ion (202-28, 94%) is formed by a loss of ethylene through McLafferty rearrangement (78) from the m/e 202 ion. The m/e 174 ion can then lose CHO to give an ion of m/e 145 (202-29, 33%). This spectrum is in good agreement with results by Skaare and Dahle (79) except that their spectrum shows an ion of m/e 144 instead of m/e 145.

The IR-spectrum shows the expected N-H stretching band at 3360 cm^{-1}. Bands at 1495 cm^{-1} and 1575 cm^{-1} are typical of aromatic compounds, and a strong band at 1255 cm^{-1} is expected for aromatic ethers.

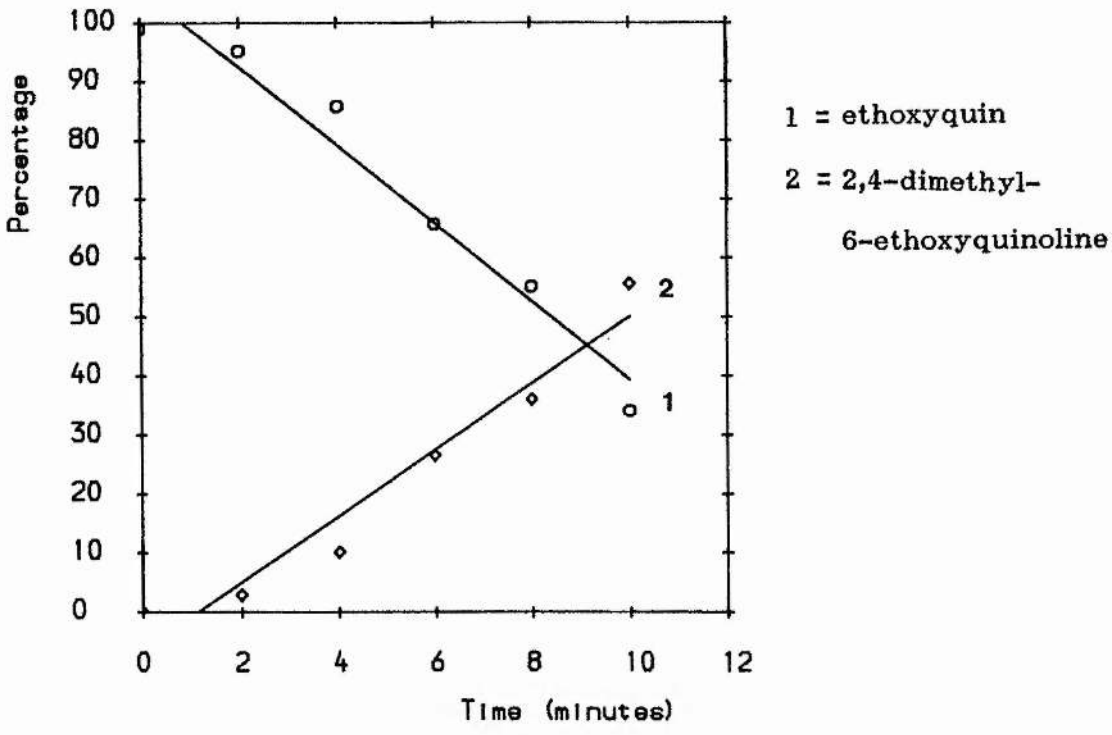
The UV-spectrum of ethoxyquin (ethanol) has absorption maxima at 229 nm (log ϵ 4.35) and 358 nm (log ϵ 3.41).

2.4 Preparation of 2,4-dimethyl-6-ethoxyquinoline

Our GC-investigation shows that ethoxyquin decomposes when heated (250 °C), to form one product almost quantitatively (Figure 2.4.1). This product was later shown to be 2,4-dimethyl-6-ethoxy-quinoline.

After heating ethoxyquin (280 °C, 13 min) a dark and viscous product was obtained. It showed two major spots by TLC; ethoxyquin (R_f 0.55) and an unknown (R_f 0.17, fluorescent). The desired product was eluted from a silica adsorption chromatography column between 2800 and 3150 ml and when the solvent was removed, light brown crystals (mp 86-87 °C, 39% yield) were obtained.

Figure 2.4.1 Decomposition of ethoxyquin at 250 °C and formation of 2,4-dimethyl-6-ethoxyquinoline.



Preparation of 2,4-dimethyl-6-ethoxyquinoline by refluxing ethoxyquin in xylene, in the presence of oxygen, gave a mixture (3.88 g) of solid and oil. After crystallisation from petroleum ether, light brown crystals (mp 86 - 87 °C, 30% yield) were obtained.

The ^{1}H-NMR spectrum was assigned as follows. Two methyl groups gave signals downfield of those in ethoxyquin, at 2.39 ppm (s, 3H) and 2.60 ppm (s, 3H), indicating a fully aromatic system. The ethoxy peaks are at 1.38 ppm ($-CH_3$, t, 3H, J ~7.5 Hz) and 4.00 ppm ($-CH_2-$, q, 2H, J ~7.5 Hz). The aromatic region peaks are at 6.97 ppm (C(3)-H, s, 1H), 7.02 ppm (C(5)-H, d, J ~3 Hz), 7.35 ppm (C(7)-H, dd, 1H, J ~3, 11 Hz) and 8.01 ppm (C(8)-H, d, 1H, J ~11 Hz). This spectrum agrees well with that of Gallagher and Stahr (80), but their interpretation of the aromatic region assigns the 7.02 ppm and the

8.01 ppm peaks to C(8)(-H and C(5)-H respectively. The ^{13}C-NMR spectrum was interpreted as can be seen in appendix 1.

The molecular ion of m/e 201 (M^+, 77%) can lose ethylene as does ethoxyquin, to form an ion of m/e 173 (M^+ -28, 100%) which by losing CHO gives m/e 144 (173-29, 15%).

The IR spectrum shows that the N-H stretching has disappeared. The UV-spectrum (ethanol) has absorption maxima at 232 nm (log ϵ 4.62), 265 nm (log ϵ 3.58), 322 nm (log ϵ 3.59) and 335 nm (log ϵ 3.61).

2.5 Preparation of 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline-nitroxide

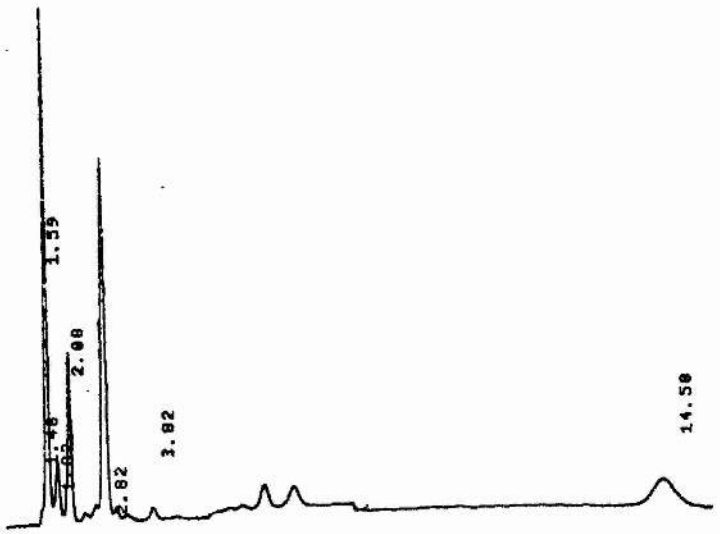
A reaction of ethoxyquin (1.0 g) with hydrogen peroxide, in the presence of sodium tungstate and EDTA, produced a dark red viscous oil (0.85 g). By TLC this oil gave four main spots; ethoxyquin (R_f 0.68), an unknown compound (R_f 0.82, fluorescent, blue), a dark red spot belived to be the nitroxide (R_f 0.58) and a yellow spot (R_f 0.23). Figure 2.5.1 shows the HPLC chromatogram for the same sample.

The peaks at 1.59, 2.08, 2.82 and 14.58 minutes correspond to the four main TLC spots. The peak at 1.59 minutes is belived to be a dimer of ethoxyquin, while ethoxyquin is eluted at 2.08 minutes and the nitroxide at 2.82 minutes. The peak at 14.58 minutes was later shown to be 2,6-dihydro-2,2,4-trimethyl-6-quinolone-N-oxide.

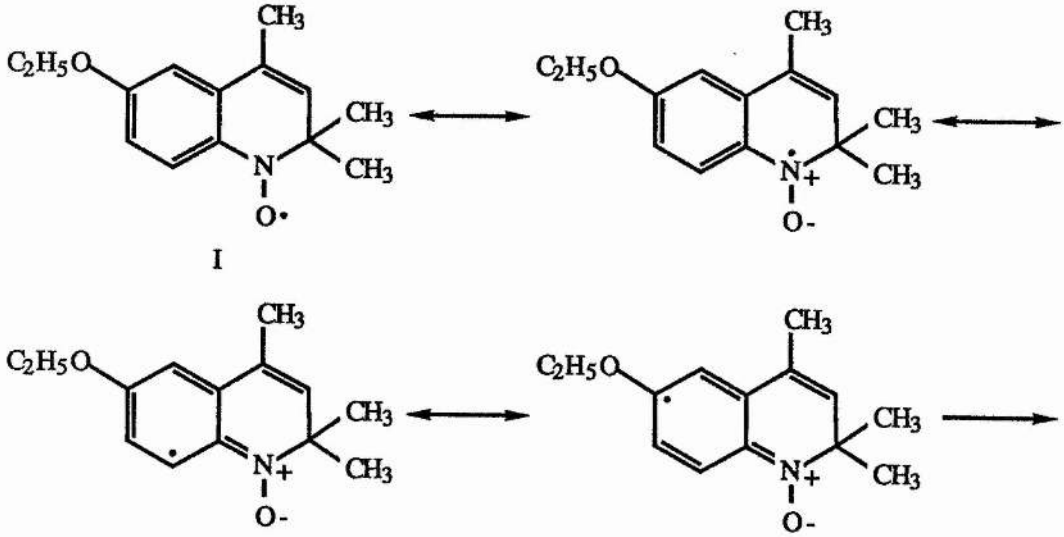
When an attempt was made to isolate the nitroxide by adsorption chromatography the nitroxide, which originally was seen as a red band, disappeared and the main product was a more polar, crystalline, yellow compound (mp 103.5 - 105.5 ^{o}C, 50% yield). This reaction also occurred on TLC plates, and the reaction product was

shown to be 2,6-dihydro-2,2,4-trimethyl-6-quinolone-N-oxide.

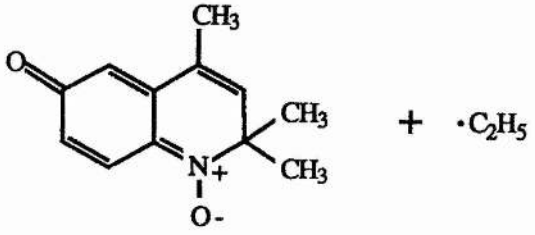
Figure 2.5.1 HPLC chromatogram of crude product from the preparation of ethoxyquin nitroxide.



It is known that when the free electron in nitroxide can delocalise in an aromatic system the stability is decreased (68). The formation of the quinolone-N-oxide (II) from ethoxyquin nitroxide (I) could be:



I



II

The three methyl groups of the quinolone-N-oxide give ^{1}H-NMR peaks at 1.60 ppm (C(2)-$(CH_3)_2$, s, 6H) and 2.07 ppm (C(4)-CH_3, d, 3H, J ~2 Hz). The four ring proton peaks are at 6.11 ppm (C(3)-H, s, 1H), 6.25 ppm (C(5)-H, d, 1H J ~2 Hz), 6.60 ppm (C(7)-H, dd, 1H, J ~2, 12.5 Hz) and 7.99 ppm (C(8)-H, d, 1H, J ~12.5 Hz).

The mass spectrum shows a molecular ion m/e 203 (55%, measured 203.0937, calculated 203.0947) which by either losing oxygen or CHO produces ions of m/e 187 (M^+ - 16, 95%) and m/e 174

(M^+ - 29, 50%). The m/e 174 ion then successively loses two methyl groups to produce ions of m/e 159 (174 - 15, 53%) and m/e 144 (159 - 15, 100%).

As expected, the IR-spectrum of the quinolone-N-oxide shows no band for the N-H stretching in ethoxyquin. Two medium strong bands at 1320 and 1220 cm^{-1} are characteristic for quinoline-N-oxides (81). The UV-spectrum (ethanol) has absorption maxima at 320 nm (log ϵ 4.25) and 378 nm (log ϵ 4.26).

Microanalysis results were 70.87% C, 6.36% H and 6.80% N: calculated for $C_{12}H_{13}NO_2$ is 70.92% C, 6.45% H and 6.89% N.

Ivanov *et al* (75), reported the formation of this compound from 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline. The ^{1}H-NMR spectrum produced by them is not unlike ours, but their interpretation is slightly different. They position the C(3)-H further downfield than the C(5)-H which disagrees with results of quinoline-N-oxide (81). The melting point of their product is 51 - 53 ^oC which is very close to our value for the corresponding quinolone (52 - 53.5 ^oC, section 2.6).

In an attempt to isolate ethoxyquin nitroxide by preparative HPLC, three main products were obtained. An unknown yellow crystalline compound believed to be a dimer of ethoxyquin, was eluted first (74 - 90 ml, 8% yield), next the nitroxide, a red amorphous solid (147 - 180 ml, 6% yield, mp 67 - 69 ^oC) and finally the main product, quinolone-N-oxide, was eluted in the last 500 ml (29% yield).

The ^{1}H-NMR for the ethoxyquin-nitroxide is typical of a free radical, the peaks are very broad and resolution very poor. The mass spectrum shows a molecular ion of m/e 232 (M^+, 70%) which by

losing three methyl groups forms ions of m/e 217 (M^+ -15, 34%), m/e 202 (217 - 15, 100%) and m/e 187 (202 - 15, 27%). The ion giving the base peak at m/e 202 can also lose the ethoxy group to give m/e 173 ion (202 - 29, 55%) and the m/e 159 ion is formed through McLafferty rearrangement from the m/e 187 ion (187 -28, 13%). Two big peaks were seen at m/e 144 (25%) and 129 (43%) and minor impurity peaks (<1%) at m/e 246 and 247. This spectrum is very different to the one published by Lin and Olcott (69) where the molecular ion peak is very small (1%).

The IR-spectrum does not include much useful information apart from the disappearance of the N-H stretching. The UV-spectrum (ethanol) has absorption maxima at 238 nm (log ϵ 4.40), 311 nm (log ϵ 3.99), 388 nm (log ϵ 3.41) and 460 nm (log ϵ 3.29). The first two wavelengths are similar to what has previously been reported, but our molar absorbtivities are about 6-9 times higher than those of Lin and Olcott (69).

Microanalysis showed the composition to be 72.03 %C, 7.76% H and 5.99% N: calculated for $C_{14}H_{18}NO_2$ is 72.38 %C, 7.81% H and 6.03%N.

Those results supported by ESR work (Chapter 3) indicate that the product is 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline-nitroxide.

2.6 Preparation of 2,6-dihydro-2,2,4-trimethyl-6-quinolone

The reaction mixture of ethoxyquin and tert-butylhydroperoxide contained three major reaction products as seen by TLC. In addition to ethoxyquin (R_f 0.54) one could see a spot, later shown to be the 1,8'-dimer of ethoxyquin (R_f 0.63), fluorescent, blue), an unknown yellow spot (R_f 0.33) and a small spot (R_f 0.20, fluorescent) thought to be 2,4-dimethyl-6-ethoxy-quinoline.

The acid extract of the mixture (red oil, 0.24 g) contained two main peaks (HPLC); ethoxyquin (1.76 minutes) and an unknown (3.98 minutes). It is interesting to see that the dimer is not extracted with the acid.

From the preparative HPLC, a yellow solid was eluted (174 - 204 ml, 15% yield, mp 52 - 53.5 ^{o}C) and when examined by analytical HPLC it contained only one peak (3.98 minutes).

In the ^{1}H-NMR spectrum, the three methyl groups give peaks at 1.40 ppm (C(2)-$(CH_3)_2$, s, 6H) and 1.98 ppm (C(4)-CH_3, d, 3H, J ~2 Hz). The ring protons give peaks at 6.32 ppm (C(3)-H, m, 1H), 6.45 ppm (C(5)-H, m, 1H), 6.62 ppm (C(7)-H, dd, 1H, J ~2.5, 12.5 Hz) and 7.17 ppm (C(8)-H, d, 1H, J ~12.5 Hz). With the help of a non-coupled spectrum, the ^{13}C-NMR spectrum was fully interpreted.

The mass spectrum shows a molecular ion at m/e 187 (52%, measured 187.0988, calculated 187.0997) which loses a methyl group to give an m/e 172 ion (M^{+} - 15, 20%, measured 172.0762, calculated 172.0762) or CO to give m/e 159 ion (M^{+} -28, 44%, measured 159.1055, calculated 159.1048). Loss of a methyl group from m/e 159 gives an m/e 144 ion (159 - 15, 100%, measured 144.0807, calculated 144.0813). The ^{1}H-NMR and the mass-spectra agree very well with results by Bonnett *et al* (43).

The IR-spectrum shows a strong band at 1635 cm^{-1} for the C=O stretching, and the absence of the N-H stretching band. The UV-spectrum (ethanol) has absorption maxima at 209 nm (log ϵ 4.40), 248 nm (log ϵ 4.12), 258 nm (log ϵ 4.12), 285 nm (log ϵ 4.18) and 364 nm (log ϵ 3.82).

Microanalysis results were 77.10% C, 7.13% H and 7.43% N; calculated for $C_{12}H_{13}NO$ is 76.99% C, 6.99% H and 7.48% N.

2.7 Preparation of 1,8'-di-(1,2-dihydro-6-ethoxy-2,2,4-trimethyl-quinoline)

A solid material (mp 107 - 109 °C, 40% yield) was obtained from the hexane extract of the acidified reaction mixture of ethoxyquin and tert-butylhydroperoxide. It was purified by preparative HPLC and fractions containing only one peak by analytical HPLC (1.75 minutes, ethoxyquin 2.20 minutes) were collected (78 - 96 ml) to give a light brown solid shown to be the 1,8'-dimer of ethoxyquin.

The ^{1}H and ^{13}C-NMR spectra were in agreement with the structure of the 1,8'-dimer.

The mass spectrum shows a molecular ion of m/e 432 (52%) which by losing three methyl groups gives ions of m/e 417 (M^+ - 15, 100%), 402 (417 - 15, 18%) and 387 (402 - 15, 13%). By losing an ethoxy group the m/e 402 ion gives an ion of m/e 373 (402 - 29, 13%). The dimer is cleaved in two ways to form an unexplained ion of m/e 215 ($M^+/2$ - 1, 6%) and an ion of m/e 201 ($M^+/2$ - 15, 56%). Those ions then both lose ethylene to give ions of m/e 187 (215 - 28, 17%) and 173 (201 - 28, 50%).

The IR-spectrum shows a very small band at 3350 cm^{-1} for one N-H stretching. The UV-spectrum (petroleum ether) has two absorption maxima at 242 nm (log ϵ 4.55) and 380 nm (log ϵ 3.73).

Microanalysis showed a composition of 77.61% C, 8.43% H and 6.42% N: calculated for $C_{28}H_{36}N_2O_2$ is 77.75% C, 8.39 H and 6.48% N.

2.8 Preparation of 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline

By refluxing ethoxyquin in hydrobromic acid, 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline is formed. The product was crystallised from toluene to give colourless crystals (mp 170 - 172 °C

(decomp.), yield 50%).

Before crystallisation, two spots were seen by TLC, the hydroxyquinoline (R_f 0.31, fluorescent, green) and an unknown (R_f 0.12, fluorescent). When spraying with aqueous ferric chloride (1%), the hydroxyquinoline spot became yellow, but no reaction was seen for the other one.

On the ^{1}H-NMR spectrum the three methyl groups give peaks at 1.20 ppm (C(2)-$(CH_3)_2$, s, 6H) and 1.91 ppm (C(4)-CH_3, d, 3H, J ~2Hz). The amino hydrogen gives a peak at 4.45 ppm (N-H, s, 1H) and the phenolic hydrogen at 7.23 ppm (-OH, s, 1H). The 3-carbon hydrogen gives a peak at 5.33 ppm (C(3)-H, d, 1H, J ~2 Hz) but the aromatic region resolution is not good enough to allow interpretation.

The mass spectrum gives a molecular ion of m/e 189 (M^+, 12.3%, measured 189.1161, calculated 189.1154) which by losing three methyl groups gives ions of m/e 174 (M^+ - 15, 100%), 159 (174 - 15, 6%) and 144 (159 -15, 8%). By losing CO from the m/e 159 ion, an ion of m/e 131 is obtained (159 - 28, 3%).

The IR-spectrum shows a small band at 3300 cm^{-1} for the O-H stretching, but the N-H stretching band is not seen because it is probably included in the nujol absorption band. The UV-spectrum (ethanol) has two absorption maxima at 227 nm (log ϵ 4.36) and 356 nm (log ϵ 3.41).

Microanalysis results were 76.59% C, 8.10% H and 7.42% N: calculated for $C_{12}H_{15}NO$ is 76.16% C, 7.99% H and 7.40% N.

APPENDIX 1 NMR SPECTRA

Table 1 ^{1}H-NMR Chemical Shift (ppm)

COMPOUND (Solvent)	N-H	C3-H	C5-H	C7-H	C8-H	(C2) $-CH_3$	(C4) $-CH_3$	$-OCH_2$ $-CH_3$	$-O$ $-CH_2-$
1[a] (CCl_4)	3.60	5.22				1.19	1.94	na	na
2[a] (CCl_4)	na	6.79	7.60		7.92	2.53	2.40	na	na
3 ($CDCl_3$)	3.42	5.45				1.25	1.98	1.37	4.05
4 ($CDCl_3$)	na	6.97	7.02	7.35	8.01	2.60	2.39	1.38	4.00
5 ($CDCl_3$)	na	6.11	6.25	6.60	7.99	1.60	2.07	na	na
6 ($CDCl_3$)	na	6.32	6.45	6.62	7.17	1.40	1.98	na	na
8[b] ($(CD_3)_2CO$)	4.45	5.33				1.20	1.91	na	na
7 ($CDCl_3$)	3.95	5.34	6.7	6.48	6.07	0.95	1.99	1.36	3.95
		5.43	6.7	6.7		1.16	2.06		
						1.22			
						1.30			

[a] Ref 76

[b] 7.23 ppm (-OH, s, 1H)

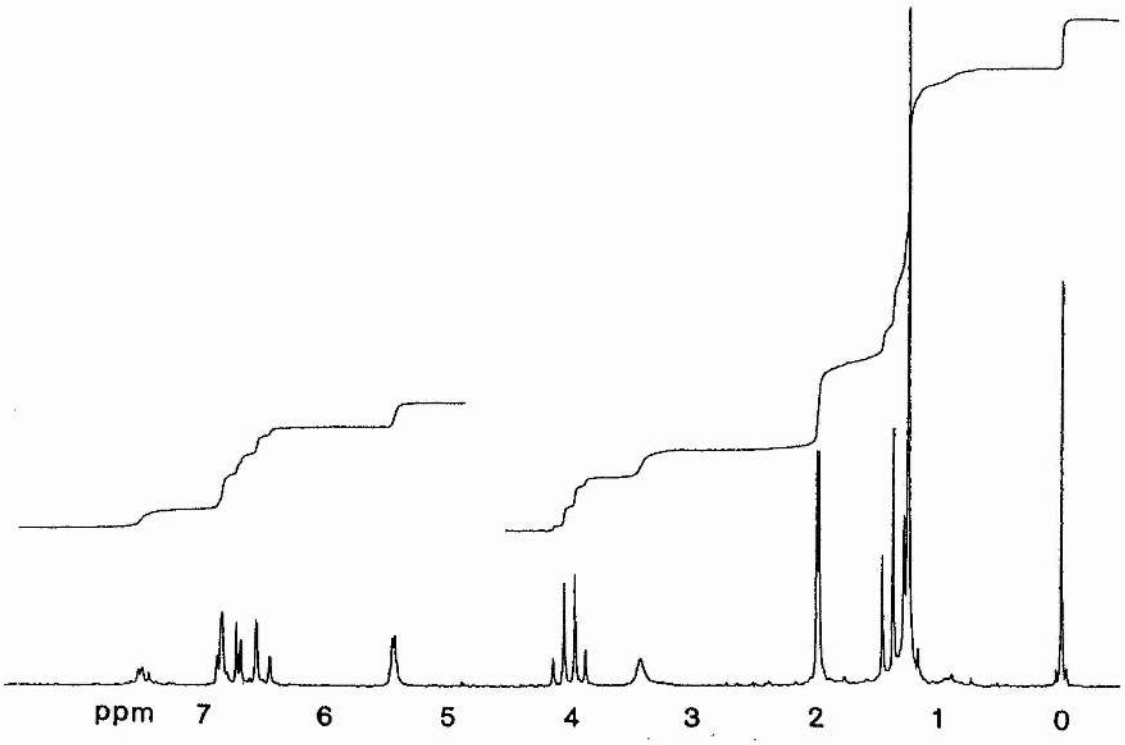
1. 1,2-Dihydro-2,2,4-trimethylquinoline (76)
2. 2,4-Dimethylquinoline (76)
3. 1,2-Dihydro-6-ethoxy-2,2,4-trimethylquinoline
4. 2,4-Dimethyl-6-ethoxyquinoline
5. 2,6-Dihydro-2,2,4-trimethyl-6-quinolone-N-oxide
6. 2,6-Dihydro-2,2,4-trimethyl-6-quinolone
7. 1,8'-Di-(1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline)
8. 1,2-Dihydro-6-hydroxy-2,2,4-trimethylquinoline
9. 2,6-Dimethylquinoline (77)
10. 6-Methoxy-2-methylquinoline (77)

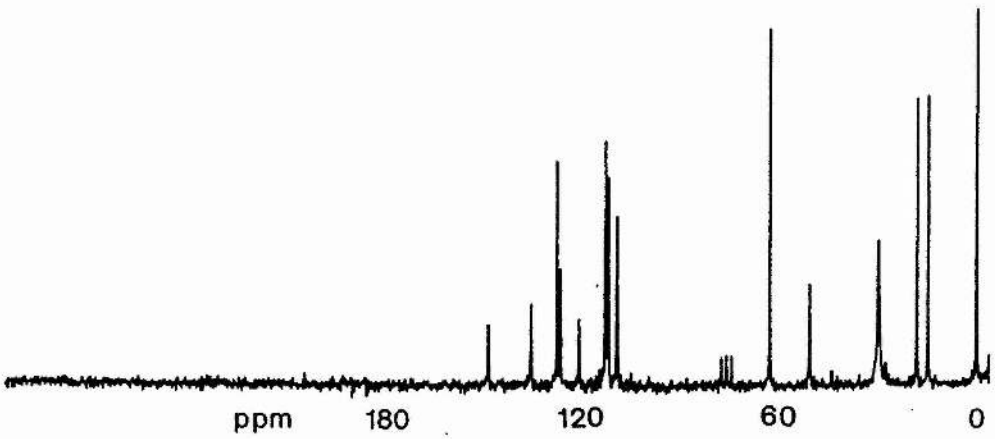
Table 2 ^{13}C-NMR Chemical shift (ppm)

COMPOUND (Solvent)		C2	C3	C4	C5	C6	C7	C8	C9	C10	C2 -C	C4 -C	-OC -C	-O -C-
9[b]	($CDCl_3$)	157.6	121.6	135.2		135.0			126.3	146.4	25.1	na	na	na
10[b]	($CDCl_3$)	155.9	121.6	134.7	105.1	157.1	121.9	129.9	127.2	143.8	24.8	na	na	55.1
3	($CDCl_3$)	51.6	111.0	128.4		151.2		129.5	122.7	137.7	30.3	18.5	15.1	64.1
4	($CDCl_3$)	155.5	122.6	142.4	102.7	156.3	121.1	130.5	127.2	143.5	24.8	18.5	14.7	63.5
6	($CDCl_3$)	61.3	119.9	126.2	145.1	187.5	132.6	140.0	130.7	186.8	28.1	17.0	na	na
7	($CDCl_3$)	51.4		128.0		150.1		117.6	123.9	137.1	27.5	18.7	15.0	63.9
		56.7		128.5		151.0		126.9	124.5	137.9	28.2	18.7	15.0	64.2
											30.2			
											30.8			

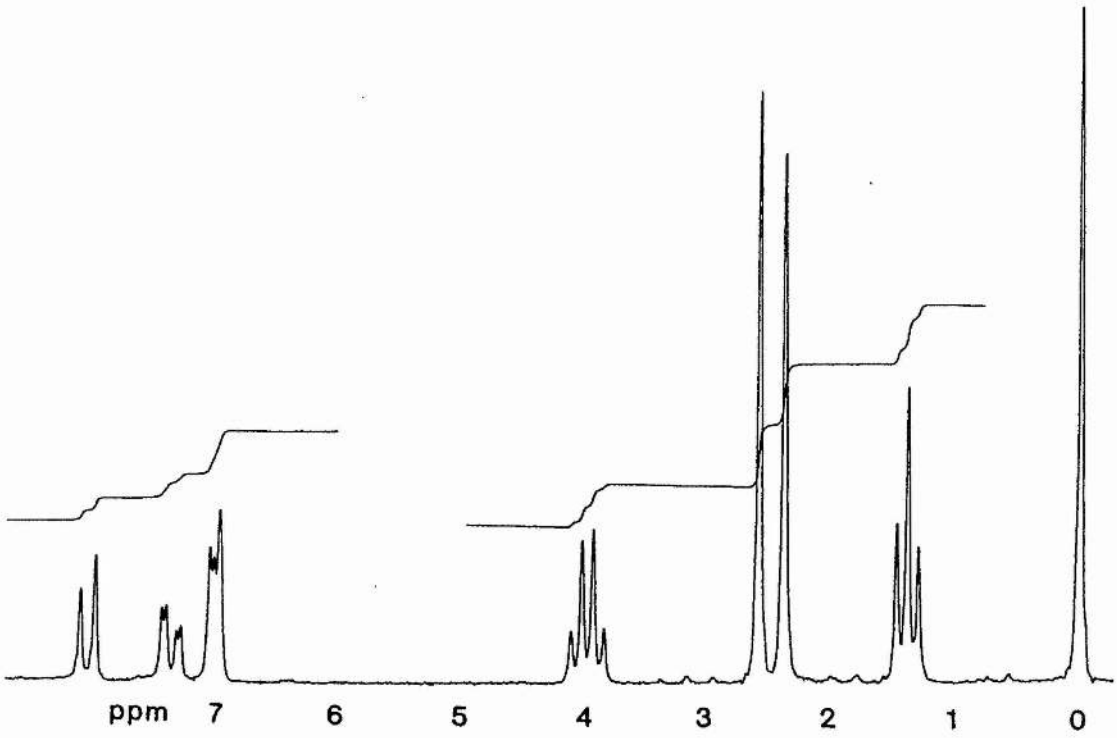
[b] Ref 77

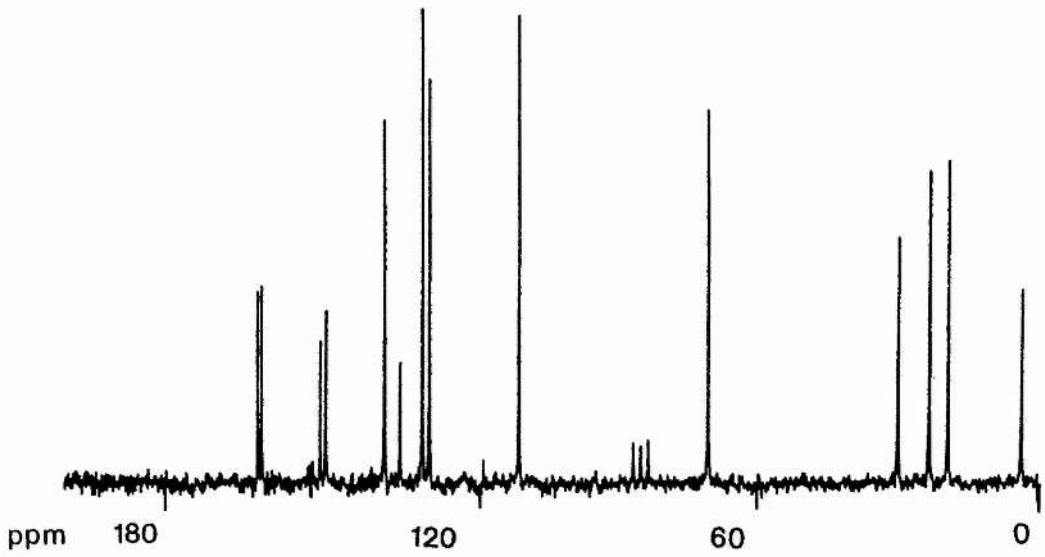
1,2-Dihydro-6-ethoxy-2,2,4-trimethylquinoline



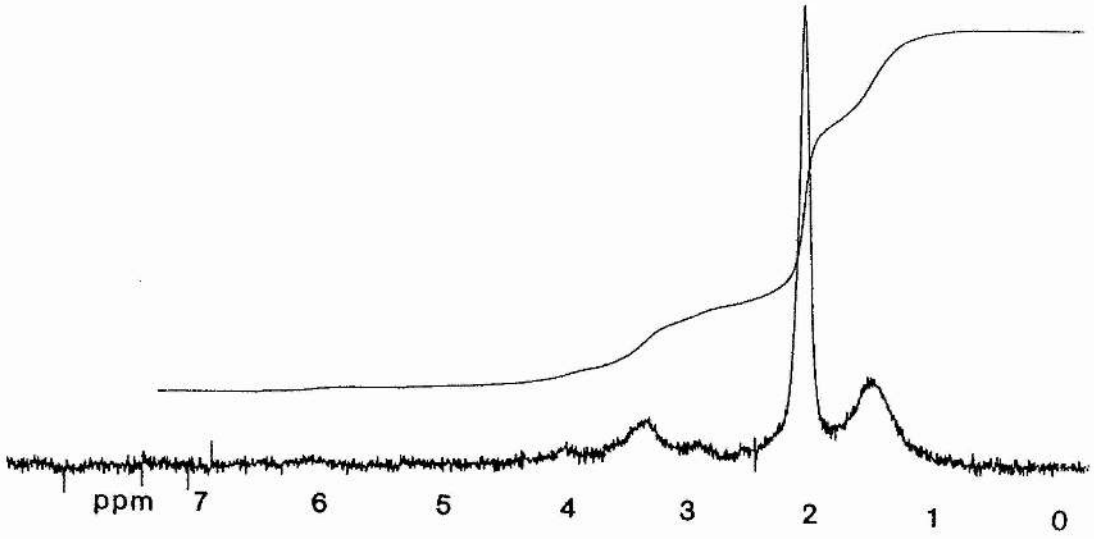


2,4-Dimethyl-6-ethoxyquinoline

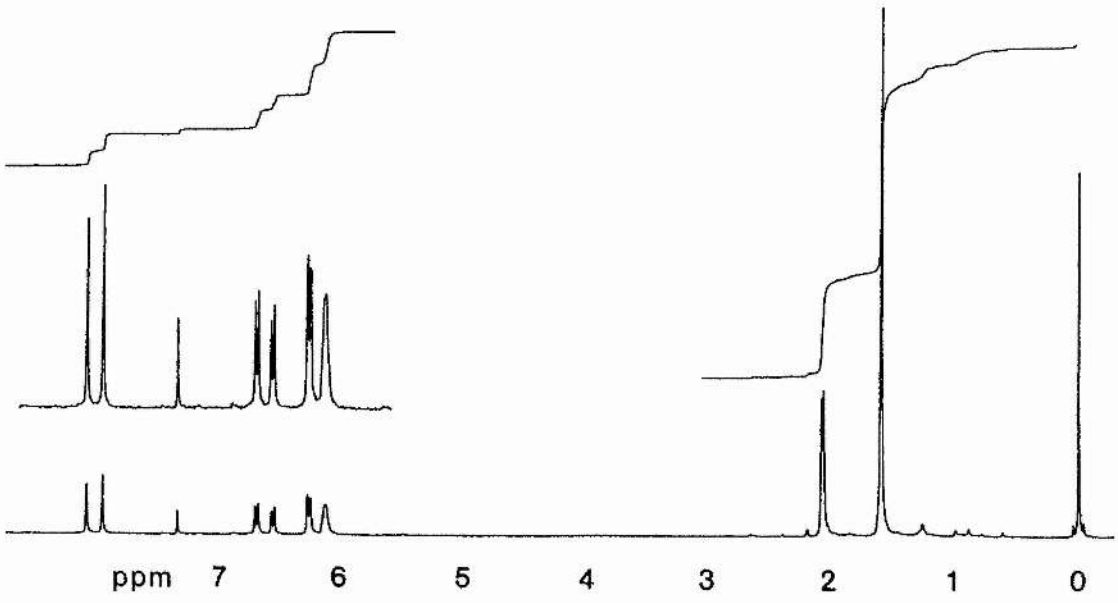




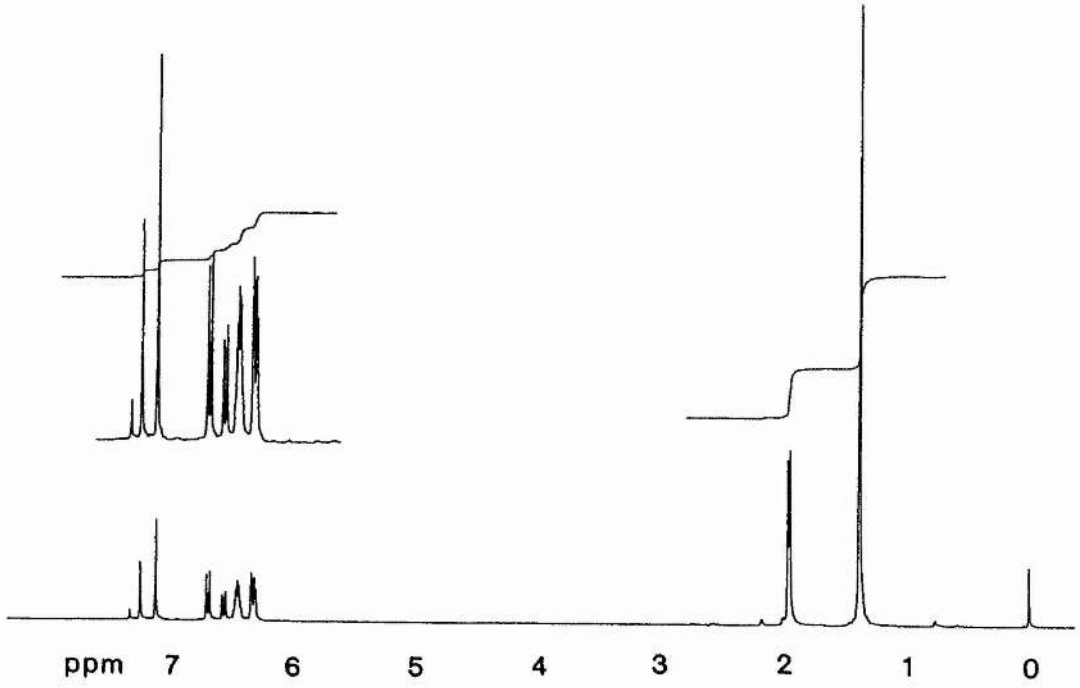
1,2-Dihydro-6-ethoxy-2,2,4-trimethylquinoline-nitroxide

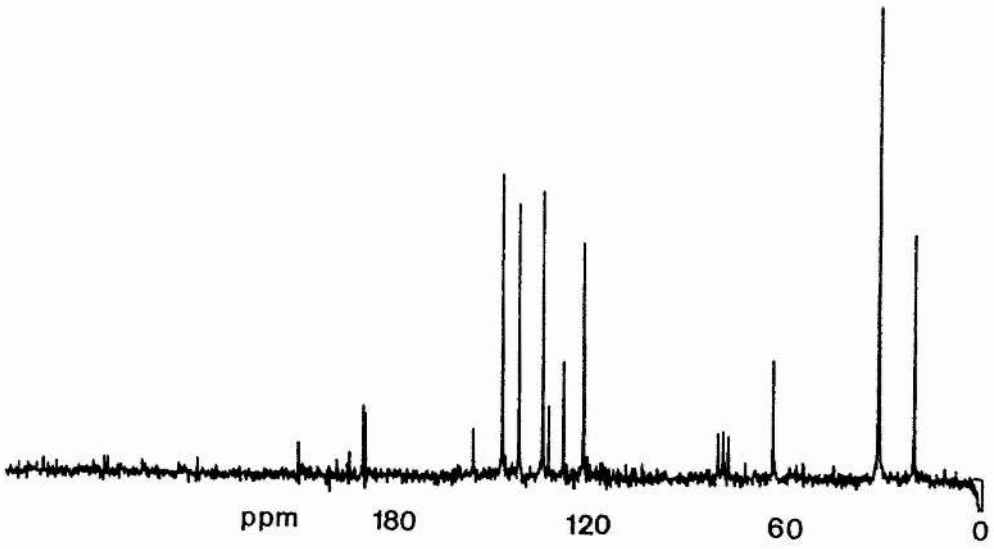


2,6-Dihydro-2,2,4-trimethyl-6-quinolone-N-oxide

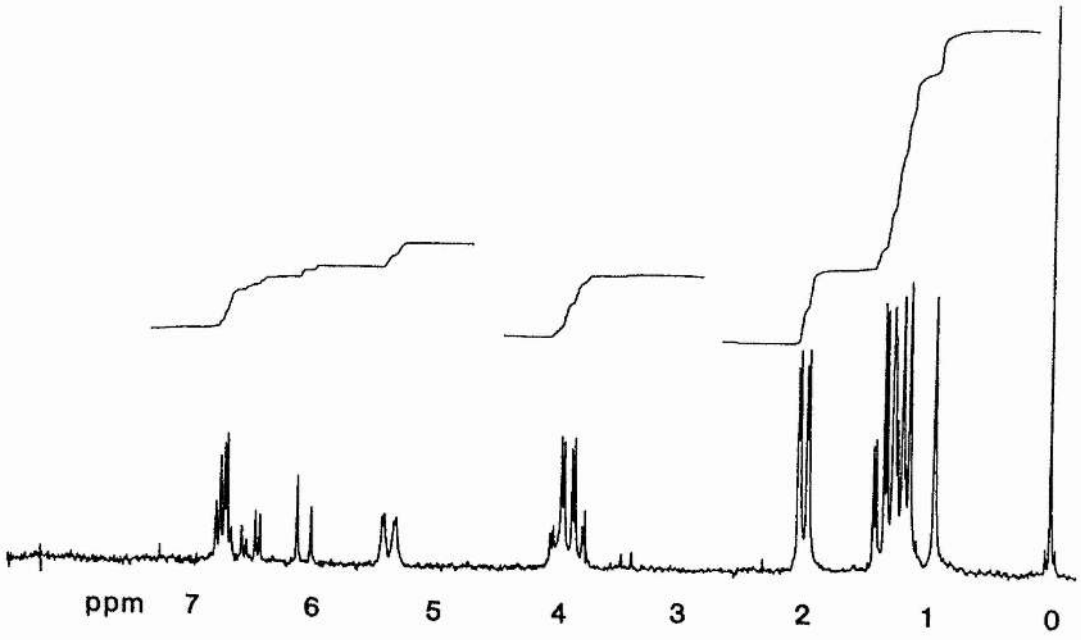


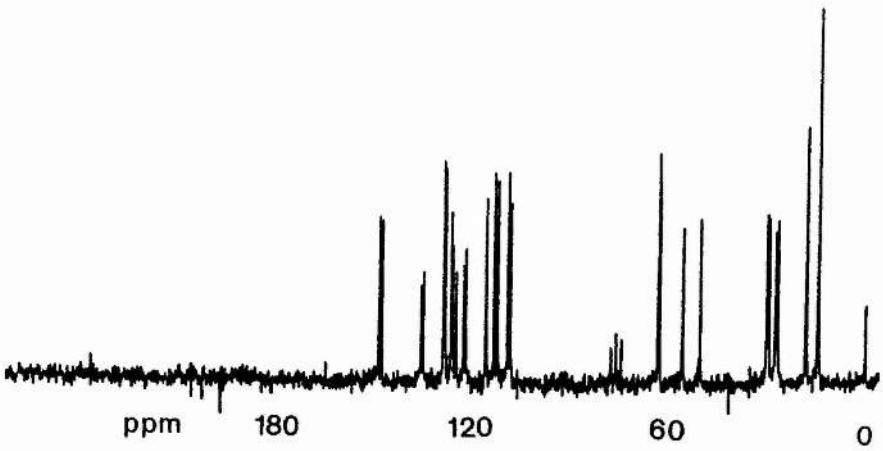
2,6-Dihydro-2,2,4-trimethyl-6-quinolone



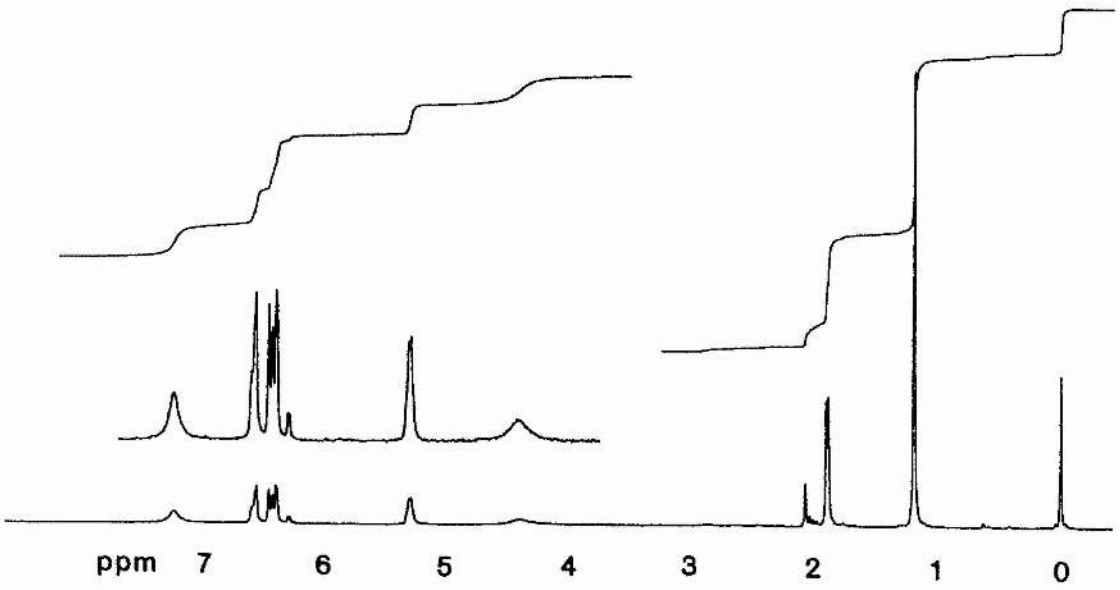


1,8'-Di-(1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline)

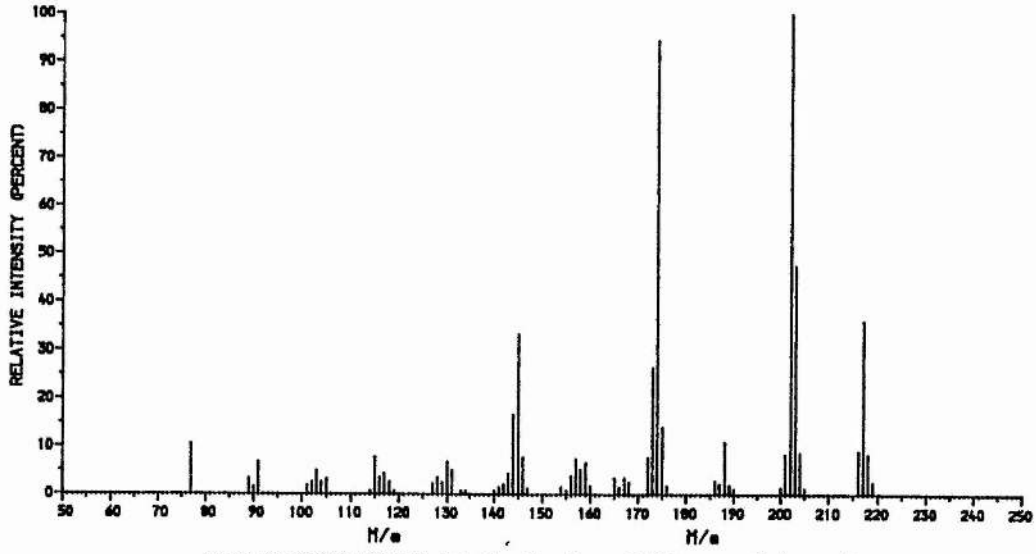




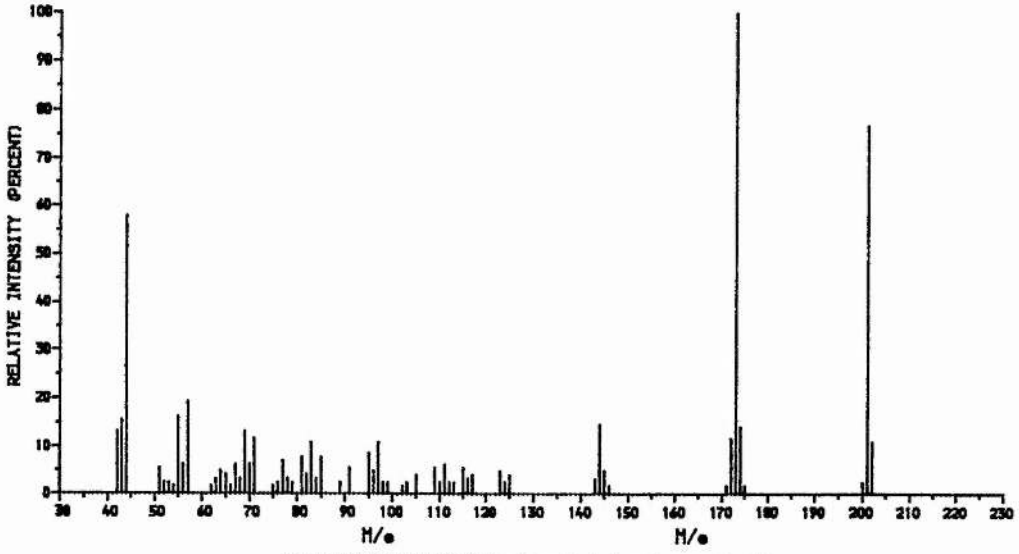
1,2-Dihydro-6-hydroxy-2,2,4-trimethylquinoline



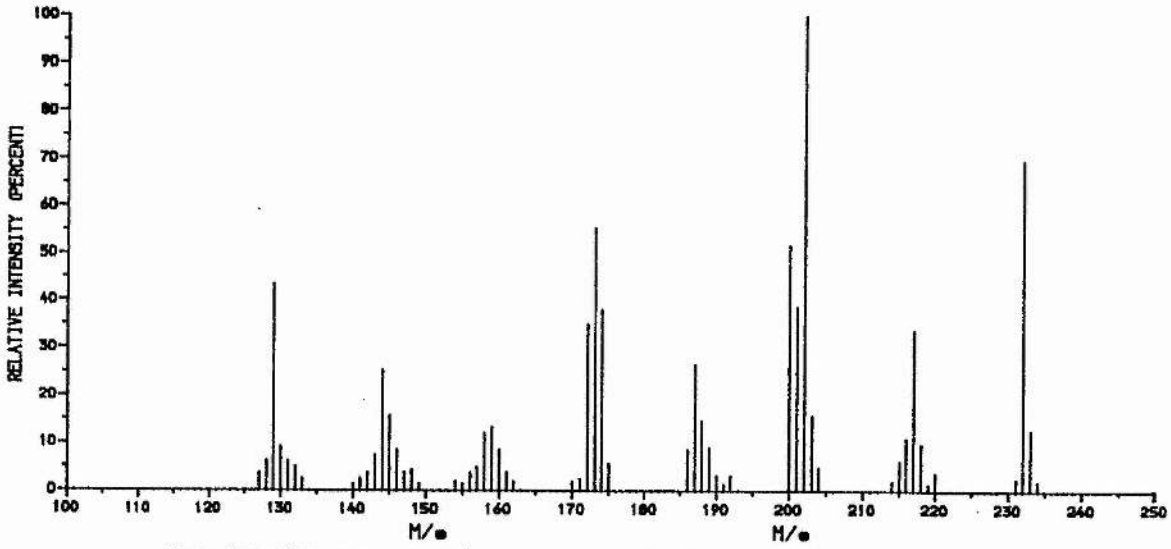
APPENDIX II MASS SPECTRA



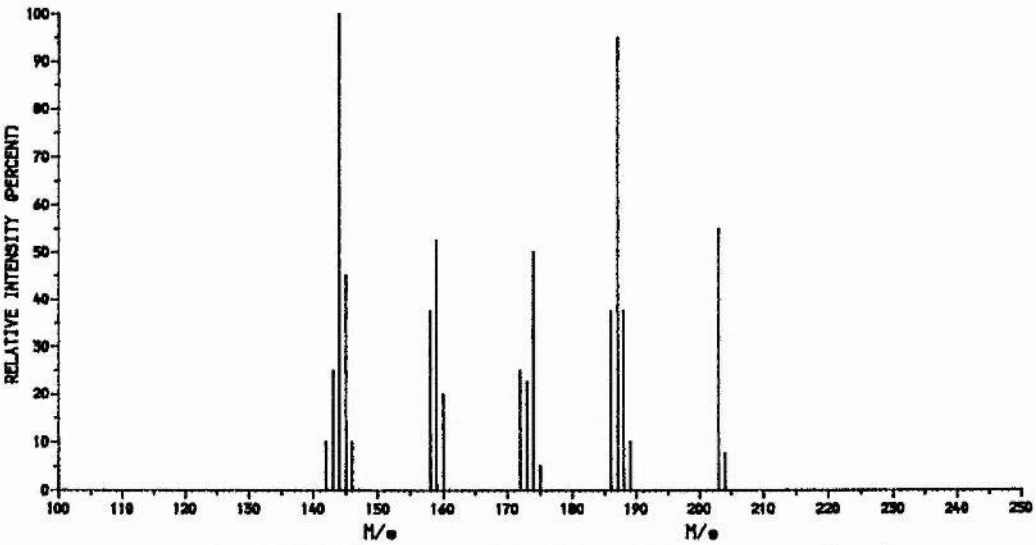
MASS SPECTRUM OF 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline



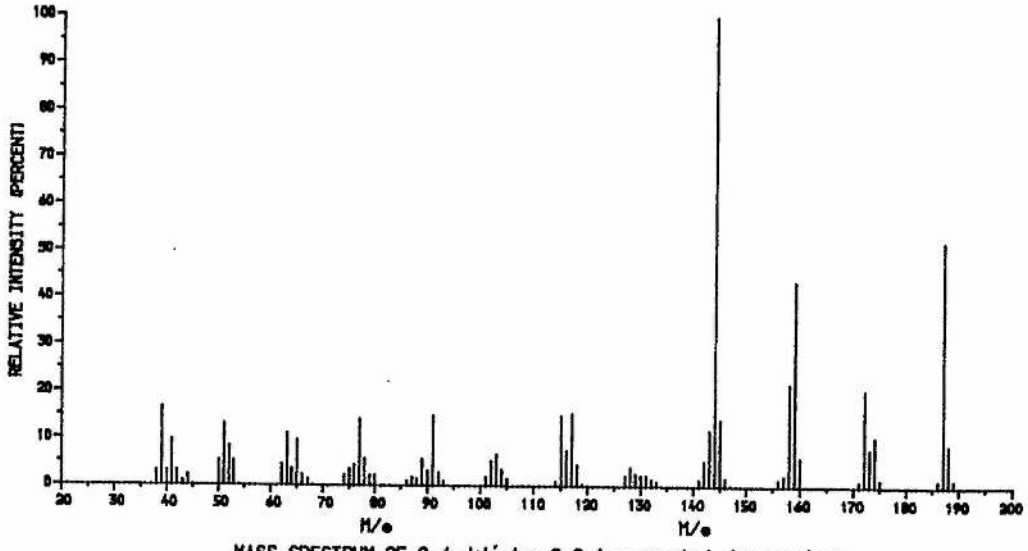
MASS SPECTRUM OF 2,4-dimethyl-6-ethoxyquinoline



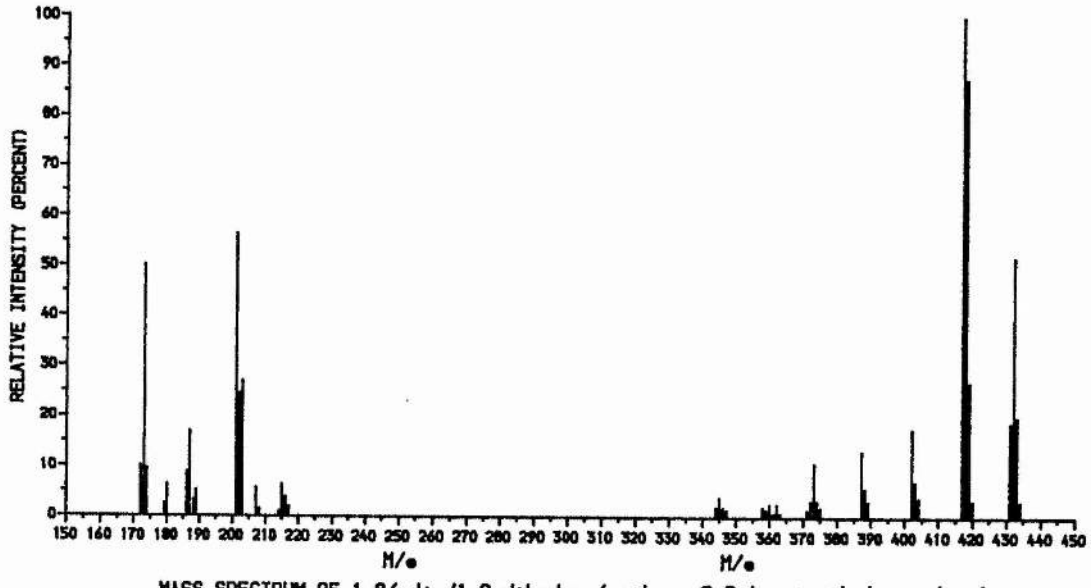
MASS SPECTRUM OF 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline-nitroxide



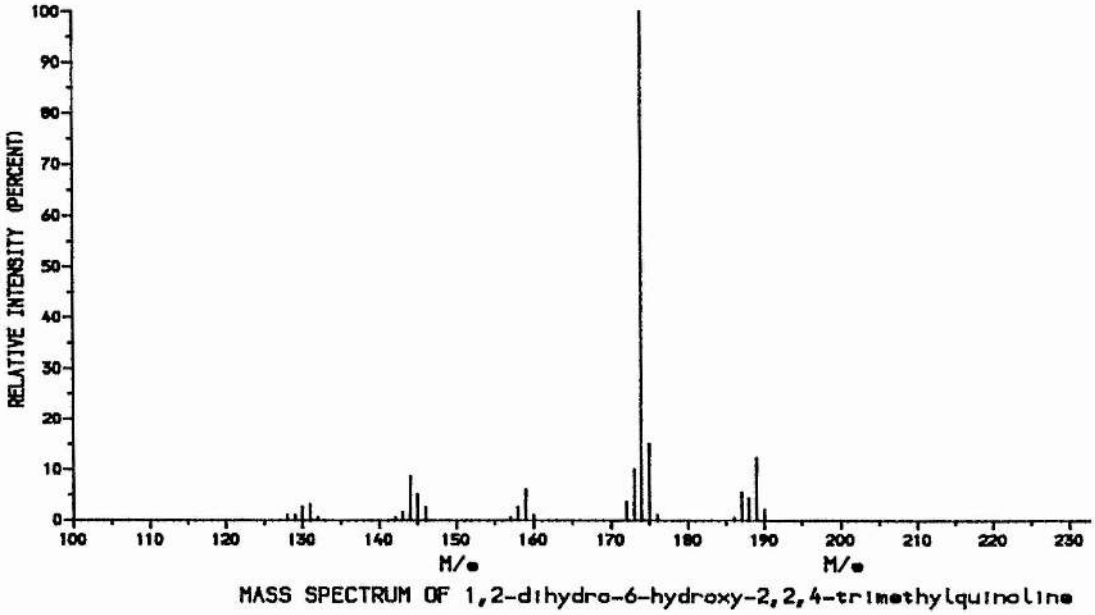
MASS SPECTRUM OF 2,6-dihydro-2,2,4-trimethyl-6-quinolone-N-oxide



MASS SPECTRUM OF 2,6-dihydro-2,2,4-trimethyl-6-quinolone



MASS SPECTRUM OF 1,8'-di-(1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline)



MASS SPECTRUM OF 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline

CHAPTER 3

ESR STUDIES OF ETHOXYQUIN AND SOME OF ITS OXIDATION PRODUCTS

3.1 Introduction

An unpaired electron has spin and a magnetic moment and, like the proton, it has a spin quantum number S = ½. The allowed spin states (2S + 1) are two and of equal energy in the absence of an external magnetic field. However, in the presence of an applied magnetic field the electron can either align itself parallel or antiparallel to the applied field. The difference in these energy states is given by,

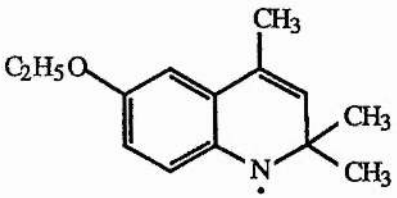
$$\Delta E = H\nu = g\beta H$$

if g is a dimensionless proportionality constant (2.0023 for the free electron), β is the Bohr magneton and H is the applied magnetic field. By applying electromagnetic radiation of changing frequency ν, the electron can move from one state to the other. In practice, the frequency is usually kept constant and the magnetic field varied.

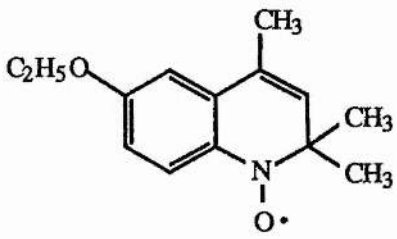
The most useful characteristic of ESR spectra is the hyperfine splitting, caused by interactions between the unpaired electron and various magnetic nuclei (^{1}H, ^{13}C, ^{14}N, etc). A proton has the spin I = ½ and with the number of nuclear spin states given as 2I + 1, the interaction of an unpaired electron with a single proton will result in a 1:1 doublet spectrum. On the other hand, an interaction with a nitrogen nucleus (I = 1) will give a 1:1:1 triplet spectrum (82, 83).

Ethoxyquin, as an antioxidant, is expected to donate a hydrogen rather easily to give the amino-radical (I). The amino-radical then can react with a peroxy-radical to give an alkoxy- and a nitroxide- radical (II) (84).

Lin and Olcott (69) and Skaare and Henriksen (85) published similar spectra obtained from ethoxyquin, consisting of a triplet of doublets, but their assignment was for the two different radicals II and I respectively.



I



II

Ethoxyquin and three of its oxidation products have now been examined by ESR in an attempt to gain knowledge about the mechanism of their antioxidant action.

Results and Discussion

3.2. ESR spectra of radicals obtained from ethoxyquin

The ESR-spectrum obtained from ethoxyquin in n-heptane or toluene (1 x 10^{-2}M) showed a triplet of doublets with an isotropic hyperfine splitting a_N 10.8 Gs and a_{H-8} 3.8 Gs. In the spectrum (Figure 3.2.1), some finer splitting is also observed. The second derivative spectrum obtained from ethoxyquin in di-tert-butylperoxide (250 K) consists of the previously seen triplet of doublets (a_N 10.8 Gs and a_{H-8} 4.1 Gs). However, when the temperature is raised (306K) a much more detailed hyperfine splitting becomes apparent (Figure 3.2.2.). This spectrum is thought to be of ethoxyquin nitroxide (II) and will be discussed further in the next section.

Different spectra from the ones previously shown were obtained by UV-irradiation of ethoxyquin solutions (Figure 3.2.3). The hyperfine splitting constants, obtained with the help of Dr R. A. Jackson, University of Sussex, are:

Group	$\underline{a}$ (Gs)
N	7.72
C(4)-CH_3	0.12
C(2)-$(CH_3)_2$	1.00
-O-CH_2-	1.01
C(3)-H	1.60
C(5)-H	2.51
C(7)-H	3.99
C(8)-H	4.11

The computer simulation is also shown in Figure 3.2.3. This spectrum is probably of the amino-radical (I) formed by abstraction of a hydrogen from the nitrogen.

It was interesting to find that at high concentrations (0.5 M), no signal was observed.

The fact that ethoxyquin is partially converted to its nitroxide in the presence of oxygen or a peroxide, supports the possibility that after abstraction of the amino hydrogen, the nitroxide can play a role in the antioxidant mechanism of ethoxyquin.

Figure 3.2.1 ESR spectrum obtained from ethoxyquin in heptane or toluene (1 x 10^{-2} M). 306 K, 9.3 GHz.

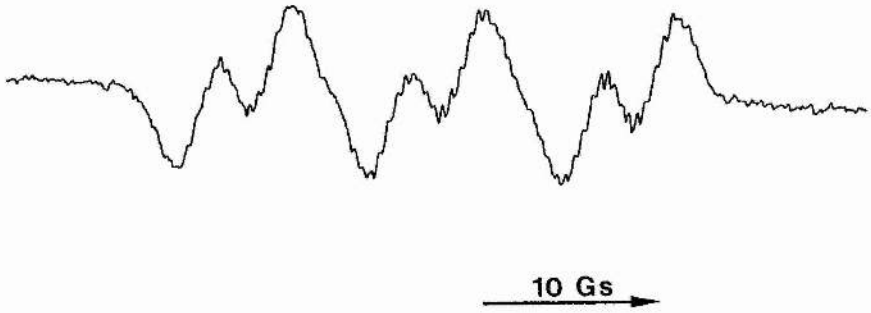


Figure 3.2.2 ESR spectrum (second derivative) obtained from ethoxyquin in di-tert-butylperoxide (1 x 10^{-2} M). 306 K, 9.3 GHz.

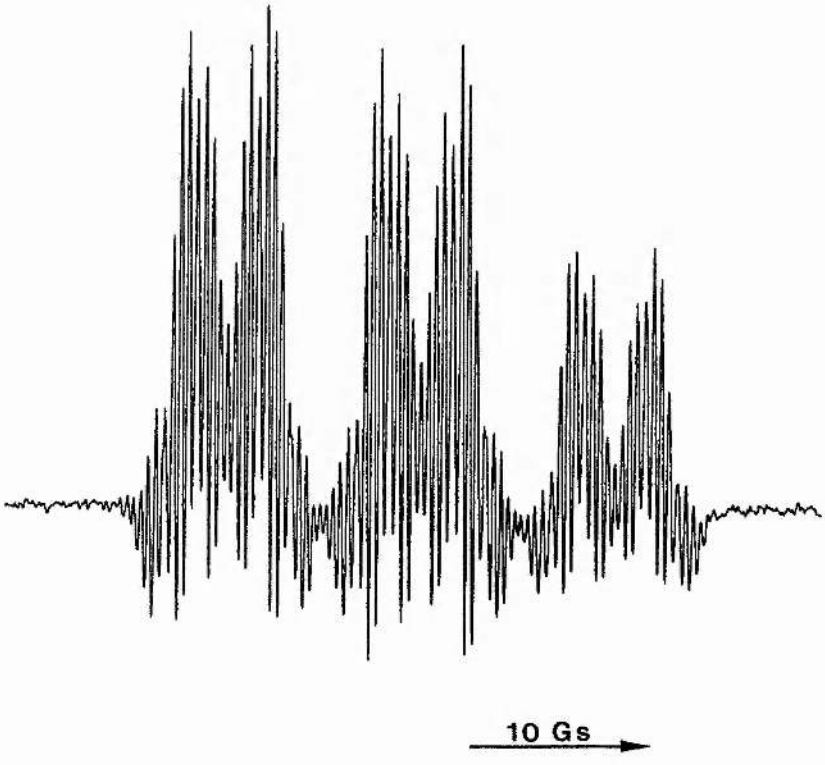
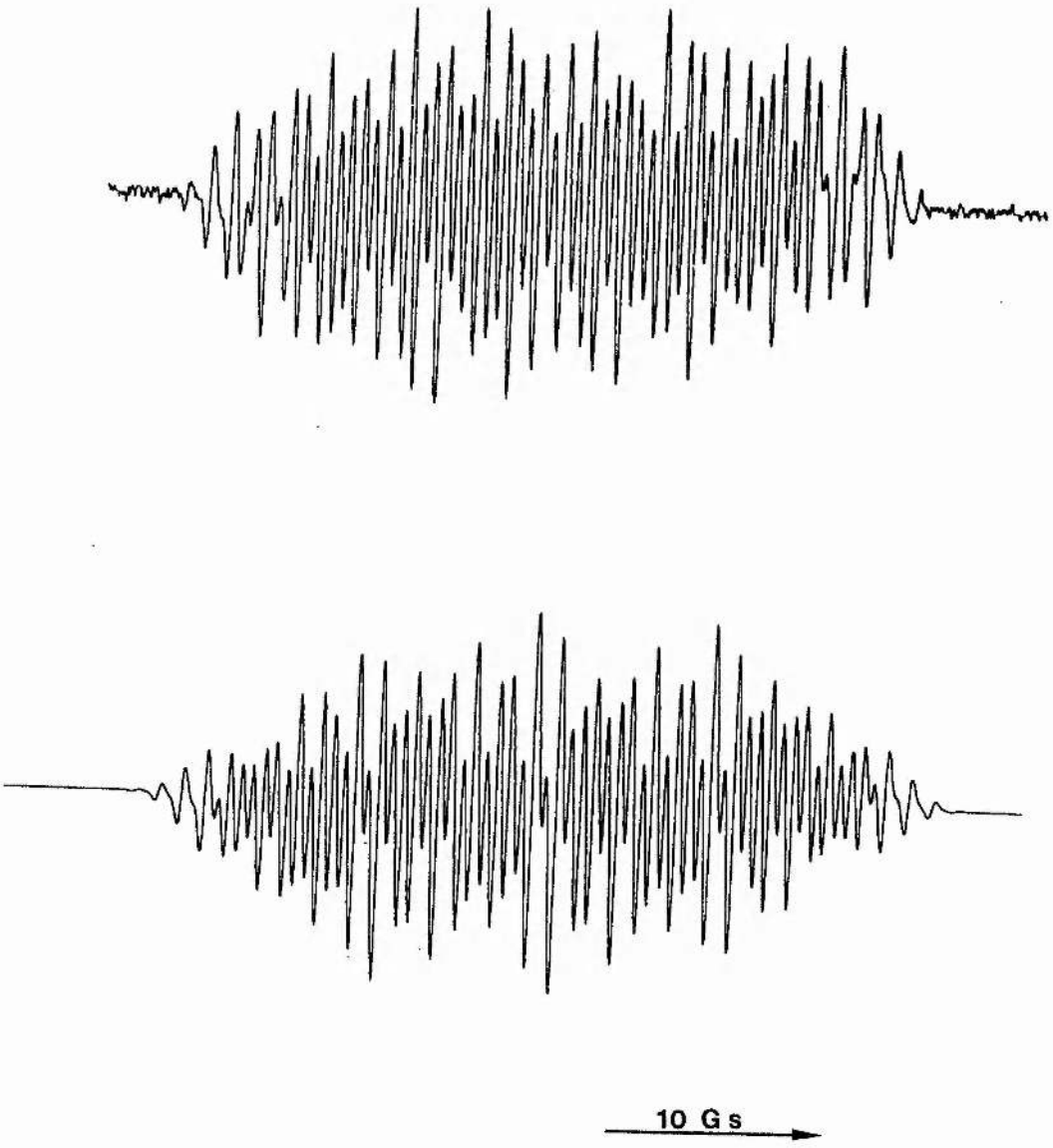


Figure 3.2.3 ESR spectrum obtained from ethoxyquin in n-heptane (1 x 10^{-2} M) after UV-irradiation. 290 K, 9.3GHz. Below is a computer simulated spectrum.



3.3 ESR spectra of ethoxyquin nitroxide

As was shown in section 3.2, the ESR spectrum of ethoxyquin nitroxide was apparent before UV-irradiation of ethoxyquin solutions.

A solution of the pure nitroxide in benzene (10^{-4} M) gave the spectrum shown in Figure 3.3.1. The triplet splitting (a_N 10.6 Gs) is similar to the previously observed values. The following hyperfine splitting constants were obtained using a computer simulation (Figure 3.3.2);

Group	a (Gs)
N	10.6
C(4)-CH_3	0.33
C(2)-$(CH_3)_2$	0.48
C(3)-H	1.01
C(5)-H	1.29
C(7)-H	1.29
C(8)-H	3.61

Figure 3.3.1 ESR spectrum (second derivative) of ethoxyquin nitroxide in benzene (1 x 10^{-4} M). 306 K, 9.3 GHz.

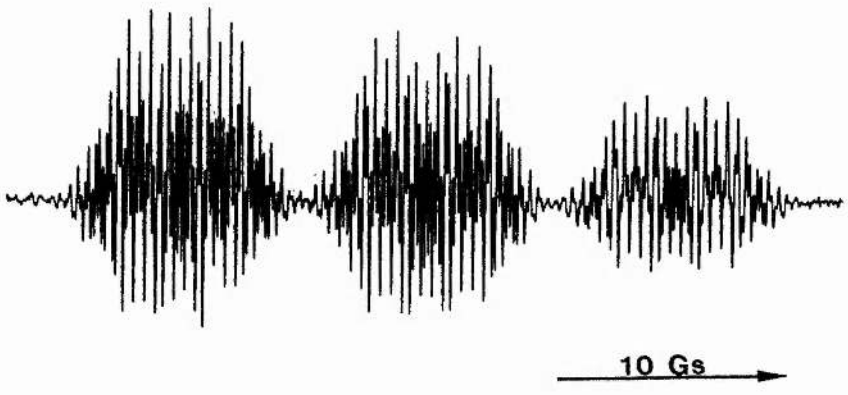
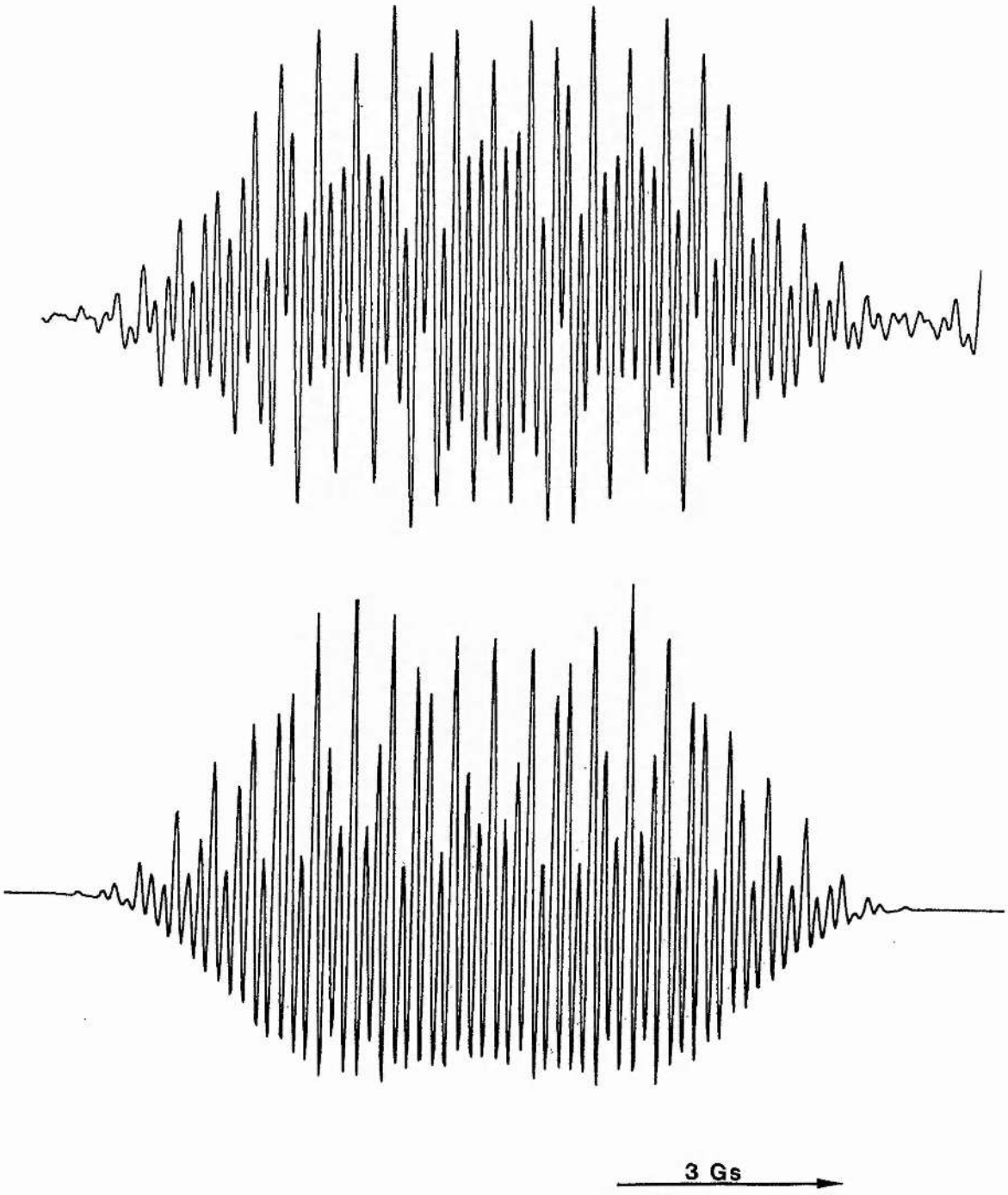
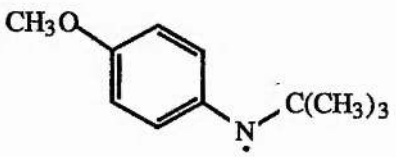


Figure 3.3.2 Part of the lowfield ESR spectrum (second derivative) of ethoxyquin nitroxide in benzene (1×10^{-4} M). 306 K, 9.3 GHz. Below is a computer simulated spectrum.



3.4 ESR spectra of radicals obtained from 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline and 2,6-dihydro-2,2,4-trimethyl-6-quinolone

A solution of the hydroxyquinoline in tert-butylbenzene (1 x 10^{-2} M) gave, when irradiated with UV-light, a spectrum thought to be derived from a mixture of the amino- and hydroxy-radicals (Figure 3.4.1a). The peaks which probably result from the amino-radical are marked, and the possible hyperfine splitting constants (a_N 4.6, a_{H-8} 9.05 Gs and a_{H-7} 4.35 Gs) give the computer simulated spectrum shown in Figure 3.4.1b. The proposed splitting constants do not compare favourably with those of amino-radicals obtained from ethoxyquin, or compounds such as tert-butyl-(4-methoxy)aminyl (III) (86).

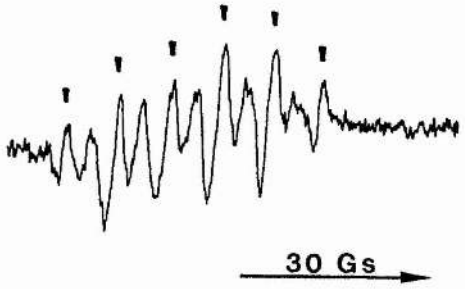


III

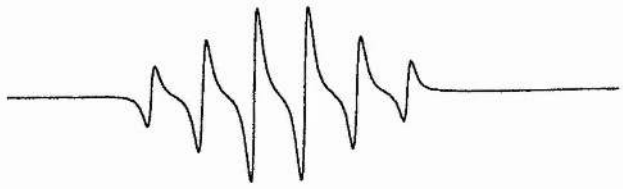
After irradiation for a few minutes, the spectrum changed (Figure 3.4.1c) and a precipitate appeared. This happened faster with the addition of a drop of di-tert-butylperoxide.

2,6-Dihydro-2,2,4-trimethyl-6-quinolone in n-heptane (1 x 10^{-2} M) gave a spectrum similar to the one in Figure 3.4.1a when UV-irradiated (Figure 3.4.2). The spectrum then changed as did the spectrum of the hydroxyquinoline, and this change was faster with the addition of di-tert-butylperoxide.

Figure 3.4.1a ESR spectrum obtained from 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline in tert-butylbenzene (1 x 10^{-2} M). 306 K, 9.3 GHz.



3.4.1b A computer simulated spectrum.



3.4.1c The spectrum after addition of di-tert-butylperoxide.

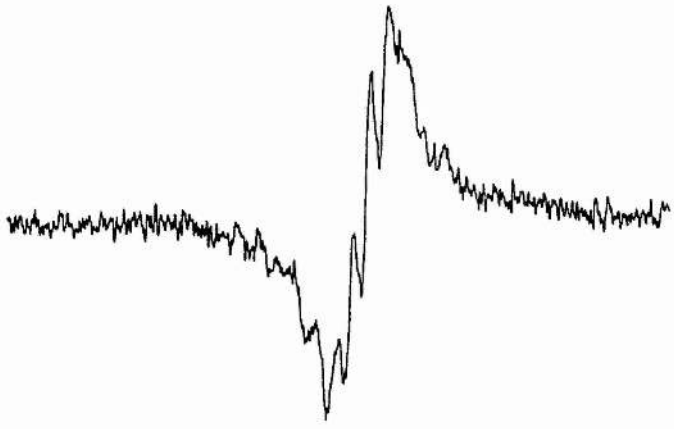
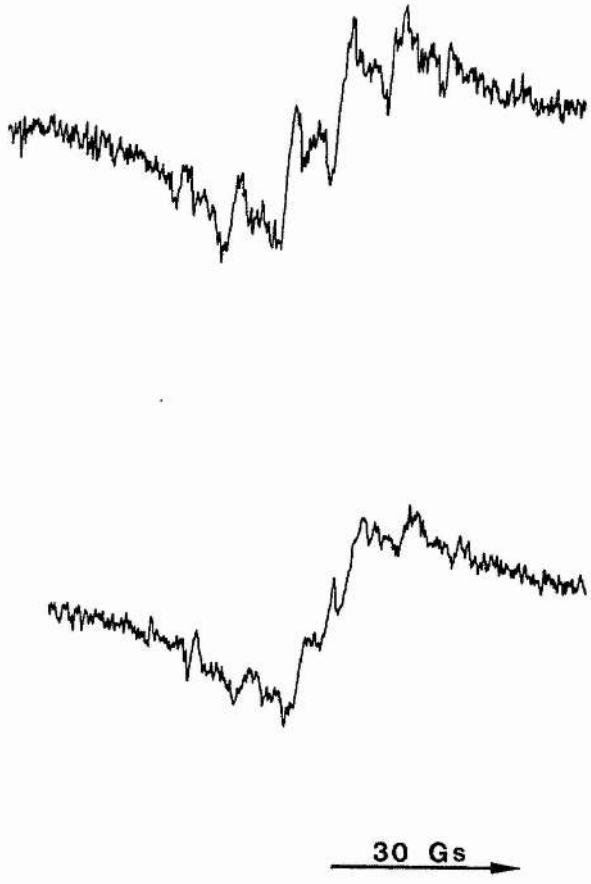
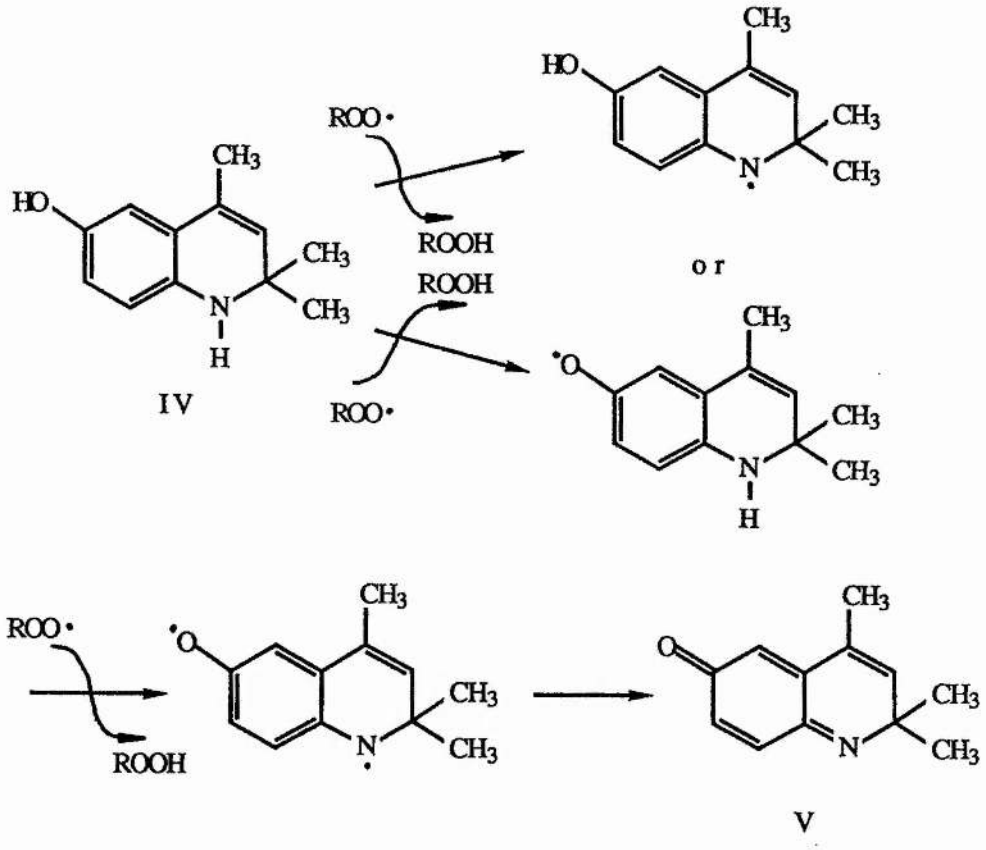


Figure 3.4.2 ESR spectrum of 2,6-dihydro-2,2,4-trimethyl-6-quinolone in n-heptane (1 x 10^{-2} M). 290K, 9.3 GHz. Below is the spectrum after addition of di-tert-butylperoxide.



A possible mechanism for the antioxidant effects of 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline (IV) could be as follows.



It seems that the 6-quinolone compound (V) can, for example when UV-irradiated change back to the di-radical form, and therefore be a good acceptor for radical addition. This is probably the explanation for the formation of the precipitate and the change of the spectrum. The products could either be di- or polymeric, or the result from addition of a tert-butoxy radical.

CHAPTER 4

LIPID ANALYSIS

4.1 Introduction

In this chapter the origin of the fish meals and oils, and their lipid analysis, are described. The oil content (chloroform extractable material) of the fish meal was measured and fatty acid analysis was carried out on these extracts and on the fish oils.

Autoxidation of the fat changes the fatty acid profile. The effect of ethoxyquin and 2,6-dihydro-2,2,4-trimethyl-6-quinolone on the autoxidation of the fatty acids and on the amount of chloroform extractables in South African fish meal are discussed in section 4.4.

All antioxidant concentrations in fish meal are given as a percentage based on the oil content, which was assumed to be 10%.

4.2 Source of experimental material

Samples of fish meal from South Africa and Chile were provided for this research through IAFMM. Both were sealed under nitrogen while still fresh. It was discovered later, that the Chilean meal contained ethoxyquin (>0.5%) which had been added in production. This meal was therefore only used in one experiment.

Samples of oils sealed under nitrogen were also provided, and a sample of fresh oil was obtained from a fish meal factory in Aberdeen. Three further samples of oil were obtained by centrifugation from frozen fresh mackerel and herring and a fourth sample (cod liver oil), extracted by the same method, was provided by the Torry Research Station.

4.3 Fatty acid analysis and oil content of fish meal

The amount of oil extracted from mackerel and two samples of herring is shown in Table 4.3.1. The two herring oils will be referred to as H1 and H2.

Table 4.3.1 Oil extraction from herring and mackerel

Source	% oil extracted
Herring (H1)	5
Herring (H2)	7
Mackerel	8

The oil content (chloroform extractables) of the South African and Chilean fish meals is shown in Table 4.3.2

Table 4.3.2 Oil contents of fish meals

Source	Oil content (%) (n = 2)
South Africa	9.66 ± 0.04
Chile	10.56 ± 0.21

Both meals have an oil content close to 10% as is assumed in the oxidation measurements.

The fatty acid composition of the oils is shown in Table 4.3.3. The amount of each fatty acid has been calculated to give a total 100%. All the samples contain a high percentage (20-40%) of

Table 4.3.3 Fatty acid analysis of fish meals and oils. % Fatty Acid (s, n = 4)

Fatty Acid	M	H1	H2	P	CO	SO	CM	SM
14:0	6.8(0.8)	8.4(0.6)	8.2(0.7)	6.5(0.8)	6.7(0.1)	11.1(0.4)	5.1(0.3)	10.9(0.3)
16:0	13.6(1.0)	15.3(0.4)	13.4(0.6)	13.5(1.0)	21.0(0.2)	18.0(0.3)	21.0(0.7)	30.8(1.2)
16:1	4.0(0.3)	7.0(0.4)	5.0(0.2)	7.7(0.9)	6.7(0.1)	12.4(0.3)	6.2(0.3)	11.3(0.3)
18:0	2.3(0.0)	1.3(0.0)	1.3(0.0)	2.0(0.2)	5.3(0.0)	3.5(0.1)	6.4(0.1)	6.3(0.3)
18:1	16.8(0.2)	11.6(0.0)	10.1(0.1)	17.2(0.1)	19.5(0.1)	11.5(0.1)	20.1(0.2)	16.2(0.5)
18:2	2.3(0.1)	1.9(0.0)	1.6(0.0)	2.2(0.4)	1.6(0.1)	1.2(0.1)	1.1(0.0)	1.1(0.1)
18:3	1.8(0.0)	1.0(0.0)	1.2(0.0)	0.9(0.0)	1.0(0.0)	0.6(0.1)	0.5(0.0)	trace
18:4	5.9(0.1)	2.6(0.0)	3.3(0.1)	3.3(0.5)	2.1(0.0)	1.7(0.1)	1.2(0.0)	0.7(0.1)
20:1	11.2(0.4)	14.4(0.3)	16.7(0.3)	14.9(0.5)	2.1(0.0)	0.9(0.0)	2.6(0.1)	1.7(0.2)
20:4	1.8(0.1)	1.1(0.1)	1.2(0.1)	1.3(0.1)	1.9(0.0)	2.1(0.1)	2.2(0.1)	0.9(0.0)
20:5	7.8(0.2)	8.0(0.1)	7.3(0.1)	7.3(0.2)	13.2(0.1)	25.4(0.4)	10.9(0.2)	8.7(0.4)
22:1	11.1(0.7)	19.1(0.7)	21.7(0.8)	13.7(1.3)	0.5(0.1)	0.7(0.0)	1.4(0.2)	1.3(0.2)
22:5	1.3(0.1)	0.8(0.0)	0.8(0.0)	0.9(0.0)	2.8(0.0)	1.7(0.1)	3.4(0.1)	0.5(0.1)
26:6	13.3(0.8)	7.3(0.3)	8.2(0.3)	8.5(0.4)	15.6(0.2)	9.1(0.3)	17.9(0.8)	9.6(0.3)
Total	100.0	99.8	100.0	99.9	100.0	99.9	100.0	100.0
% of oil	77.3(5.5)	79.9(3.2)	72.5(2.0)	80.8(5.2)	71.9(0.7)	69.9(1.7)	64.6(3.1)	44.3(1.7)

s = standard deviation

M = Mackerel oil
H = Herring oil
P = Aberdeen fish meal-plant oil
CO = Chilean oil
SO = South African oil
CM = Chilean meal
SM = South African meal

polyunsaturated fatty acids which makes them susceptible to autoxidation. In all the samples except the South African meal, the fatty acids measured are between 65 and 80% of the oil. This together with the fact that the amount of polyunsaturated fatty acids is much lower in the South African meal than in the Chilean meal, suggests prior oxidation which would not happen to the Chilean meal, because it already contained ethoxyquin.

Although not obvious from these results it was considered, from their colour and smell, that the oils from the fish meal plants were of inferior quality to the other oils.

4.4 Effects of antioxidants

The oil content and the fatty acid composition were measured in fish meal after 18 weeks incubation at 30 °C. The results are shown in Tables 4.4.1 and 4.4.2.

Table 4.4.1 Oil content of fish meal

Antioxidant	Oil (%) (s, n = 2)
None	8.9 (0.5)
EQ 0.1%	9.5 (0.4)
EQ 0.5%	10.0 (0.0)
QLO 0.1%	9.6 (0.3)
QLO 0.5%	10.2[a]

s = standard deviation

EQ = ethoxyquin

QLO = 2,6-dihydro-2,2,4-trimethyl-6-quinolone

[a] only one sample

Table 4.4.2 Fatty acid composition of fish meal after autoxidation. % Fatty Acid (s, n = 4)

Fatty Acid	Control	EQ 0.1%	EQ 0.5%	QLO 0.1%	QLO 0.5%[a]
14:0	13.1(0.2)	11.7(0.2)	10.3(0.1)	11.9(0.3)	10.4(0.0)
16:0	33.2(0.3)	30.2(0.3)	27.0(0.1)	30.6(0.4)	27.2(0.0)
16:1	13.4(0.1)	12.2(0.1)	11.1(0.4)	12.4(0.2)	11.5(0.4)
18:0	6.6(0.0)	6.1(0.1)	5.5(0.0)	6.0(0.1)	5.6(0.0)
18:1	18.4(0.1)	16.9(0.1)	15.3(0.1)	16.9(0.2)	15.4(0.0)
18:2	1.2(0.1)	1.3(0.1)	1.3(0.0)	1.5(0.2)	1.3(0.1)
18:3	trace	trace	0.4(0.0)	trace	trace
18:4	trace	0.9(0.1)	1.1(0.0)	1.2(0.2)	1.1(0.0)
20:1	1.8(0.0)	1.9(0.1)	1.8(0.0)	1.8(0.1)	1.7(0.1)
20:4	trace	0.8(0.1)	1.2(0.1)	0.9(0.1)	1.1(0.0)
20:5	6.4(0.3)	9.3(0.2)	12.1(0.2)	8.7(0.7)	12.2(0.1)
22:1	1.6(0.2)	1.5(0.1)	1.4(0.1)	1.4(0.1)	1.4(0.0)
22:5	trace	0.6(0.1)	1.0(0.1)	0.4(0.1)	1.0(0.0)
22:6	4.3(0.1)	6.6(0.2)	10.4(0.4)	6.3(0.5)	10.0(0.2)
Total	100.0	100.0	99.9	100.0	99.9
% of oil	41.8(1.6)	44.1(2.4)	45.5(1.4)	43.9(0.5)	45.0(0.8)

EQ = ethoxyquin
QLO = 2,6-dihydro-2,2,4-trimethyl-6-quinolone
[a] n = 2

The oil content (chloroform extractable material) decreases over the 18 week period of storage at 30 °C, unless an antioxidant is present. This is because oxidised oil is less soluble in chloroform, and/or is bound to other components of the meal (87, 50).

In Table 4.4.2 it can be seen that the polyunsaturated fatty acids are equally well protected by both antioxidants. The bottom row shows the 14 fatty acids as a percentage of the chloroform extracts, and it is slightly higher for higher concentration of antioxidants. The lipid analysis for the meal containing 0.5% antioxidants indicate that the sample of original meal was slightly oxidised before analysis.

If the samples containing 0.5% antioxidants are taken to have the original fatty acid composition, the relations between fatty acid loss and oxygen uptake in the other samples can be obtained (Table 4.4.3). If a reaction of only one mole of oxygen is assumed with each fatty acid, then it can be shown from the results that the oxygen uptake is 11-18 times bigger than can be explained by a single peroxidation of the lost fatty acids.

Table 4.4.3 Oxygen uptake and loss of fatty acids in fish meal

Antioxidant	Oxygen uptake (μmole/g oil)	Loss of fatty acids (μmole/g oil
EQ 0.1%	2069 (±135)	109
None	2556 (±207.5)	227
EQ 0.5%	776 (±63)	-

Waissbluth *et al* (88), explained their similar results in terms of

the reaction of the fatty acids with more than one mole of oxygen. However, if all double bonds of the lost fatty acids would react with oxygen, the total oxygen uptake is still higher than the loss of fatty acids. This indicates the presence of some other reactions involving uptake of oxygen.

The 14 measured fatty acids were only 45% of the extractable oil. The rest, other lipid material and extractable oxidation products, in addition to the more polar (non-extractable) peroxidised lipids, can probably contribute significantly to the oxygen uptake. Although chloroform extracted fish meal showed no tendency to react with oxygen (88), Zirlin and Karel (89) found that in the presence of linoleate, gelatine undergoes various changes, possibly due to radical oxidation of the protein, initiated by linoleate peroxy-radicals.

CHAPTER 5

AUTOXIDATION OF FISH MEAL AND FISH OIL

5.1 Introduction

Methods for the determination of lipid autoxidation can be divided into three groups. Determination of:

a) Primary or secondary reaction products

b) Decrease in substrate concentration

c) Oxygen uptake

The primary autoxidation products of unsaturated fatty acids are allylic hydroperoxides (5). They and most other peroxides react with potassium iodide in acid solution to liberate iodine, which then can be titrated with thiosulphate. In the later stages of the autoxidation, this method becomes less useful because the peroxides break down to form various secondary products.

In the formation of hydroperoxides from polyunsaturated fatty acids, conjugated dienes are formed (3). Measuring the increase in absorption at 233 nm can therefore be a useful method of following the progress of oxidation. In complex systems like fish meal however, other components are likely to cause interference.

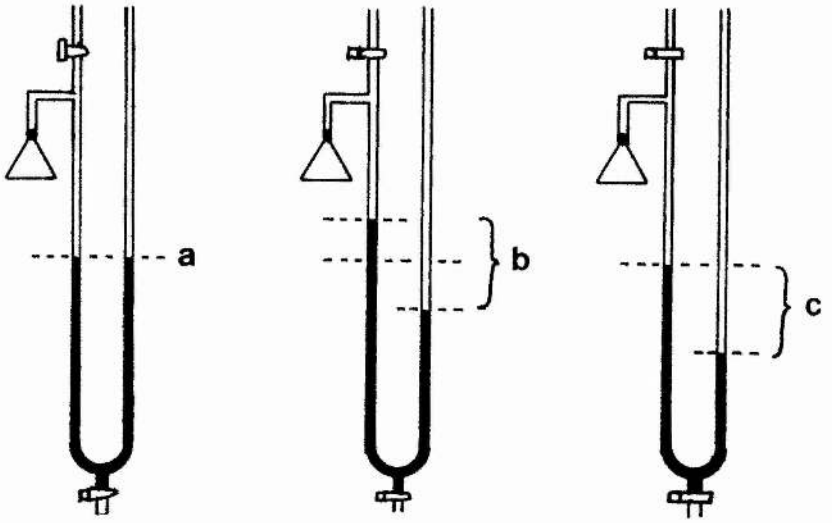
Chain fission of the hydroperoxides leads to the formation of a variety of lower molecular weight products. Malondialdehyde is a secondary autoxidation product of fatty acids with three or more double bonds and in the TBA (thiobarbituric acid) test the absorption at 532 nm is measured. This is the absorption maximum for the reaction products of malondialdehyde and 2-thiobarbituric acid (90). One disadvantage of this method is that other aldehydes and oxidized protein can react with 2-thiobarbituric acid to give products absorbing at the same wavelength (91).

Amongst other methods, based on secondary product formation, are colourimetric determination of total carbonyl compounds as their 2,4-dinitrophenylhydrazones, and aldehyde and pentane analysis by GC (90).

Various GC-systems have been used to measure the decrease of certain unsaturated fatty acids in autoxidising systems.

Oxygen uptake can be determined either by measuring changes in gas composition above a sample in a closed vial, or by manometric techniques. The Warburg constant volume technique which is described by Umbreit *et al* (92) measures the pressure decrease in a flask of a known volume. This method is widely used for autoxidation measurements in fish meal, and oxygen uptake measurements have been strongly recommended by various workers, for products such as freeze dried meats (93).

Figure 5.1.1 Warburg manometer operation



The procedure for the Warburg oxygen uptake measurements is illustrated in Figure 5.1.1. At the start of the experiment the columns of manometric fluid are adjusted to a certain mark (a). Uptake of oxygen is seen as a difference in height in the two columns (b), the flask side column is adjusted to the mark (constant volume) and the reading taken (c). Changes in atmospheric pressure and small variations in temperature are measured and corrected for by use of an empty flask connected to a manometer.

Two assumptions are made:

a) Pressure decrease is only due to reactions of the lipids and oxygen.
b) No gases such as carbon dioxide are being evolved from the sample.

In fish meal, some protein oxidation may occur, but at very low level compared to the lipid autoxidation. Waissbluth *et al* (88) stated that after solvent extraction, fish meal showed no tendency to absorb oxygen. However, Zarlin and Karel (89) suggested that lipid-peroxide initiated protein oxidation occurred in model systems.

Bacterial growth in the sample can cause carbon dioxide formation which would affect the results. This is not a problem in oils, and the low water activity in fish meal should inhibit bacterial growth. However, it has been shown that some carbon dioxide does evolve from fish meal (94), but this is not significant below 60 ^{o}C.

Oxygen uptake (μmol/g oil) = P x k

where

$$k = (V - w/d) \times \frac{273 \times 10^6}{H \times w_l \times T \times 2.24 \times 10^4}$$

and P = pressure change (mm)

V = volume of flask and manometer (ml)

w = weight of sample (g)

w_l = weight of lipid (w = w_l for oil)

d^a = density of sample (g/ml)

T = experimental temperature (K)

H = 760 mm Hg

[a] estimated 1.4 g/ml for fish meal (95) and 0.9 g/ml for oils.

The main advantage of the Warburg manometric technique is its accuracy and sensitivity, nor does it measure reaction products which are greatly dependent on the substrate system composition. The sensitivity in most of the experiments described in this chapter, allows detection of oxygen uptake equal to peroxide values of less than one.

One disadvantage is that even if the overall rate of lipid autoxidation is independent of oxygen pressure at moderate pressures (7), this is not so in fish meal as shown by Davidovitch *et al* (96) and Quarst and Karel (97). The unsteady state diffusion of a gas into a plate of product can be described by:

$$D \frac{\partial^2 c}{\partial x^2} - \frac{\partial c}{\partial t} - kc = 0$$

where D = gas diffusitivity (cm^2/s)

x = distance (cm)

c = concentration ($moles/cm^3$)

t = time (s)

^{a}k = reaction rate constant (s^{-1})

If k and c are low, the diffusion process can be represented by Fick's law of diffusion;

$$D \frac{\partial^2 c}{\partial x^2} = \frac{\partial c}{\partial t} \qquad (98)$$

In the South African meal, which had big particle size, the diffusion is probably dependent both on diffusion into the bulk and then into each particle.

Because the rate of autoxidation in the meal is dependent on oxygen concentration, it is important to make frequent air exchange in the flask. It is also necessary to realise that factors such as ratio of sample size to flask volume will affect the rate of oxygen uptake.

[a] first order reaction with respect to oxygen is expected

5.2 Ethoxyquin in fish oils

When herring oil (H1) was autoxidised (30 °C) in the presence of ethoxyquin at different concentrations, the oxygen uptake was linear against time for at least 140 hours. The rate of oxygen uptake is shown in Table 5.2.1.

Table 5.2.1 Autoxidation of herring oil (30 °C), rate of oxygen uptake over 140 hours.

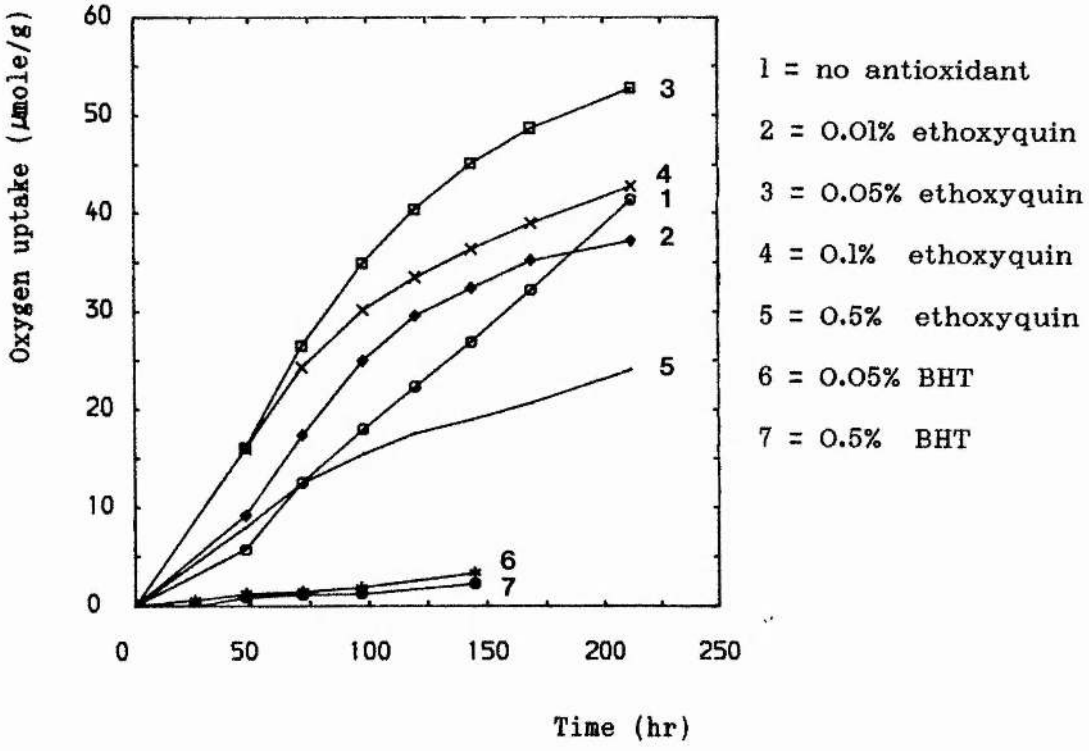
Ethoxyquin concentration(%)	Rate of oxygen uptake (μmol/g hr)	Corr. Coeff.
0.0	0.19	0.993
0.0	0.18	0.996
0.01	0.21	0.998
0.01	0.19	0.997
0.05	0.24	1.000
0.05	0.21	0.993
0.05	0.22	0.995
0.1	0.16	0.998
0.1	0.16	0.995
0.5	0.07	0.996
0.5	0.06	0.996
0.5	0.06	0.991

The lines are calculated from points after 20 hours to avoid effects from short inhibition periods in some of the samples. These results indicate that whilst the rate of oxygen uptake is reduced by ethoxyquin at concentrations of 0.1 and 0.5%, it is increased at lower concentrations (0.01 and 0.05%). Similar results were obtained (Figure 5.2.1) when this experiment was repeated, but the rates

decreased towards the end of the experiment.

The effect of BHT in herring oil was compared to the effect of ethoxyquin and as can be seen in Figure 5.2.1, they are very different. There is little difference between samples containing different concentrations of BHT. Both have the same low oxygen uptake rate (0.02 μmole/g hr) whilst the samples containing ethoxyquin have 9-15 times faster initial oxygen uptake.

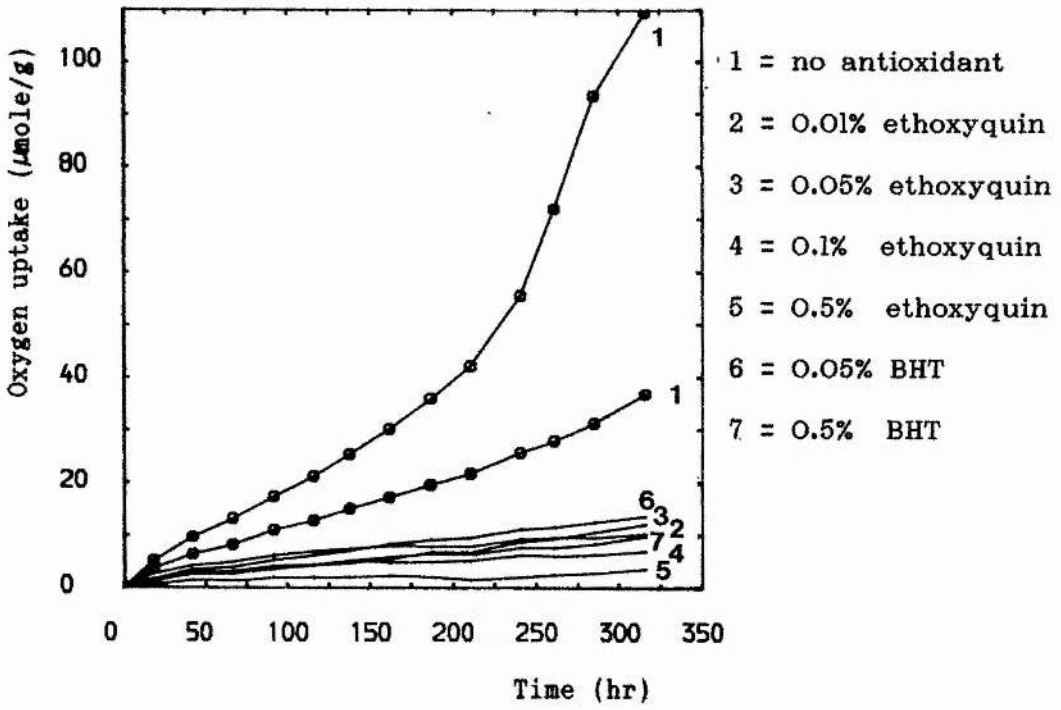
Figure 5.2.1 Autoxidation of herring oil (30 °C), oxygen uptake vs time.



In an oil obtained from a fish meal plant, ethoxyquin showed similar effects to BHT and slightly better activity (Figure 5.2.2). Compared to the herring oil, this oil is very crude, judging from its

colour and smell. In this oil, ethoxyquin has immediate effect at all concentrations.

Figure 5.2.2 Autoxidation of oil from a fishmeal plant (30 °C), oxygen uptake vs time.



For an unknown reason, one control autoxidized much more slowly than the other.

Olcott (99) observed that ethoxyquin was a more effective antioxidant than propyl gallate or NDGA (nordihydro-guairetic acid) in different crude fish oils. On the other hand, ethoxyquin and NDGA were equally effective in refined cod liver oil and NDGA was a more powerful antioxidant than ethoxyquin in lard. Furthermore, he noticed that by adding free fatty acids into oil, the adverse effects on the influence of the antioxidants were greater with NDGA than

with ethoxyquin. It is known that fatty acids, combined as triglycerides, autoxidise more slowly than their methyl esters, which are slower than the free acids (7, 100, 101). Not only do free acids autoxidise more readily, they also catalyse autoxidation. This was shown by Miyashita and Takagi (102), where on adding stearic acid, which does not autoxidise, into soybean oil, the rate of autoxidation increased. They also showed that those effects were probably due to the hydroperoxides breaking down faster in the presence of the free acid to form either alkoxy- or peroxy-radicals.

As with the oil from the fish meal plant, ethoxyquin reduced the oxygen uptake immediately in the Chilean and South African oils. After inhibition period of approximately 70 hours, the Chilean oil takes up oxygen at a rate 0.7 μmol/g hr compared to approximately 0.05 and 0.1 μmole/g hr for the samples containing 0.5 and 0.1% ethoxyquin respectively (Figure 5.2.3). The South African oil has a slightly faster oxygen uptake (Figure 5.2.4), the control having no inhibition period. There is also very little difference between the two concentrations of ethoxyquin.

Both the Chilean and South African oils were autoxidised in duplicate, and to give an idea about the reproducibility the average end point and the error for each sample is given in Table 5.2.2.

It is possible that when ethoxyquin reacts with oxidation products of the oil, it forms another compound which is a more potent antioxidant. Therefore, in a low quality oil which is already oxidised, ethoxyquin reacts quickly and further autoxidation of the oil is inhibited, whereas in a better quality oil some oxygen uptake takes place before ethoxyquin or its derivative starts having any effect.

Figure 5.2.3 Autoxidation of Chilean oil (30 °C), oxygen uptake vs time.

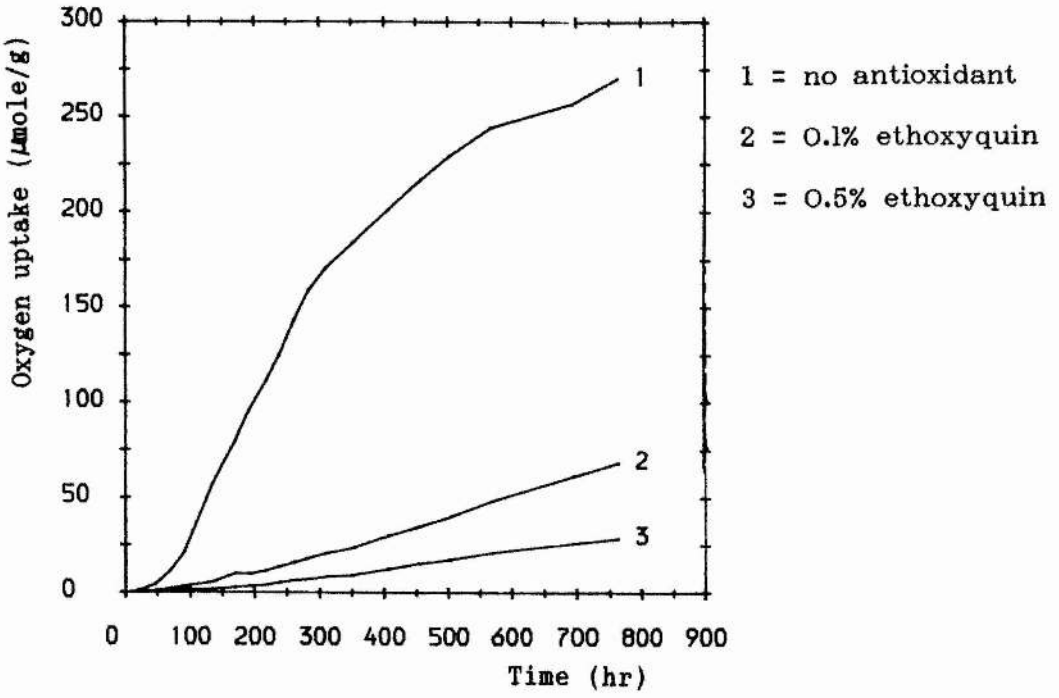


Figure 5.2.4 Autoxidation of South African oil (30 °C), oxygen uptake vs time.

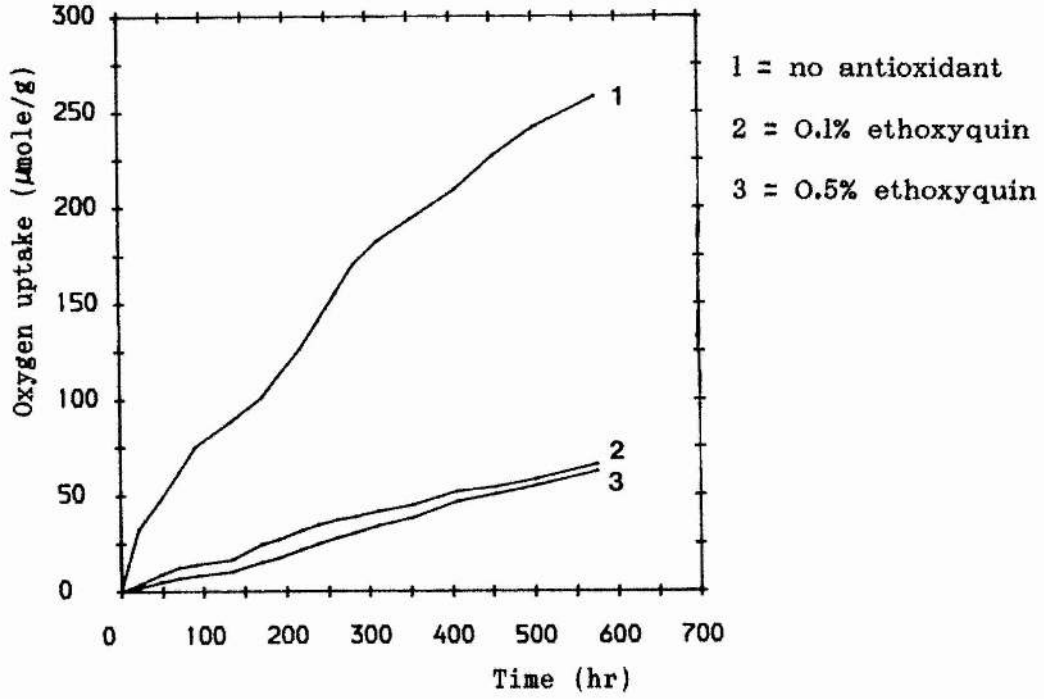
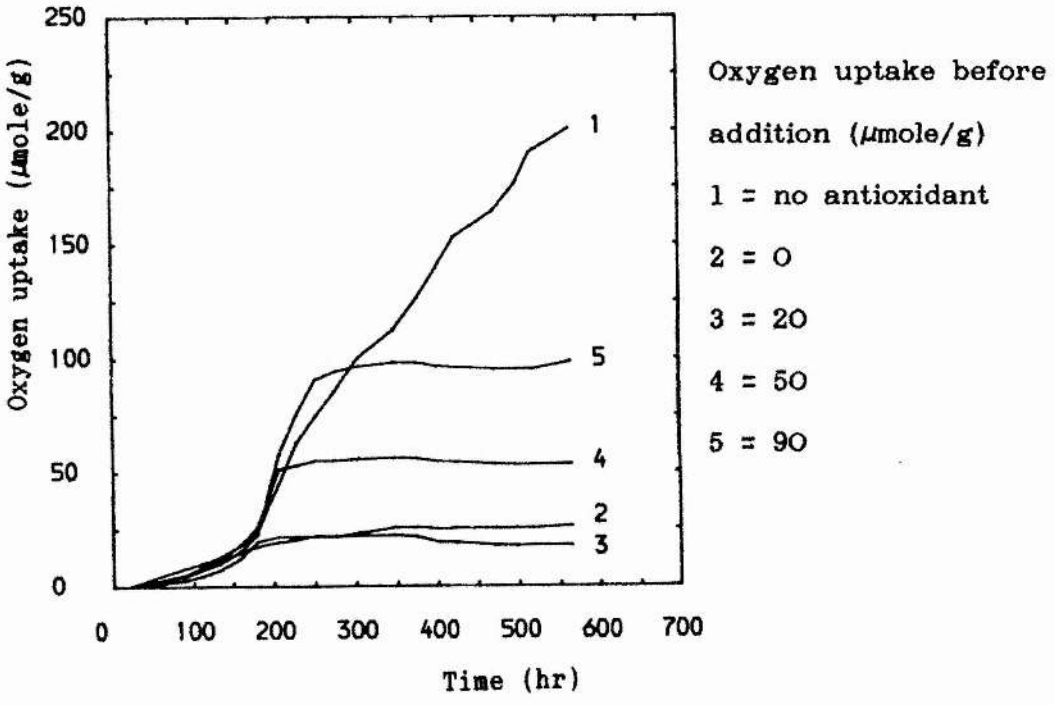


Table 5.2.2 Oxygen uptake for Chilean and South African oil.

Oil	Ethoxyquin concentration (%)	Oxygen uptake (µmol/g)	Error (±)
Chilean	0.0	270.2	24.9
Chilean	0.1	68.4	1.1
Chilean	0.5	28.7	1.5
South African	0.0	258.3	0.7
South African	0.1	65.9	1.7
South African	0.5	62.5	4.2

Figure 5.2.5. shows the autoxidation of mackerel oil when 0.1% ethoxyquin is added at different levels of oxygen uptake.

Figure 5.2.5 Autoxidation of mackerel oil (30 °C), ethoxyquin (0.1%) added at different levels of oxygen uptake. Oxygen uptake vs time.



Some oxygen uptake occurs before any antioxidant effects are seen, and it reaches approximately 25 μmole/g before it stops. However, when ethoxyquin is added after some oxygen has reacted with the fish oil (20, 50 and 90 μmole/g), the oxygen uptake stops very quickly. This points to a very significant conclusion: Lipid autoxidation products, most likely the peroxy-radicals convert ethoxyquin to one or more products with antioxidant properties. With fresh (un-autoxidised) oil, ethoxyquin promotes autoxidation for a short initial period. This is most noticable at low ethoxyquin concentrations.

5.3 Ethoxyquin in fish meal

Adding ethoxyquin to the Chilean fish meal had no effect on the oxygen uptake (Figure 5.3.1). All the samples had the same rate of oxygen uptake (0.13-0.14 μmole/g hr), which decreased slightly later in the experiment. As was mentioned previously (section 4.2), it was later discovered that the meal already contained ethoxyquin (>0.5%), so it is obvious that addition above this level has no further effects and that some oxygen uptake always takes place.

Figure 5.3.1 Autoxidation of Chilean fish meal (30 °C), oxygen uptake vs time.

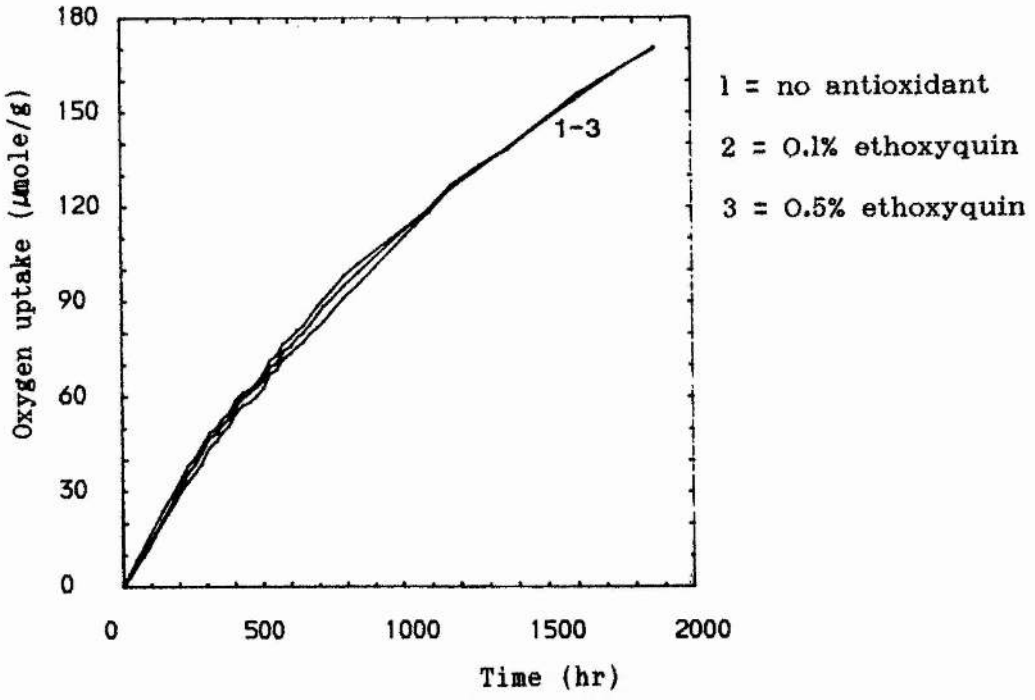
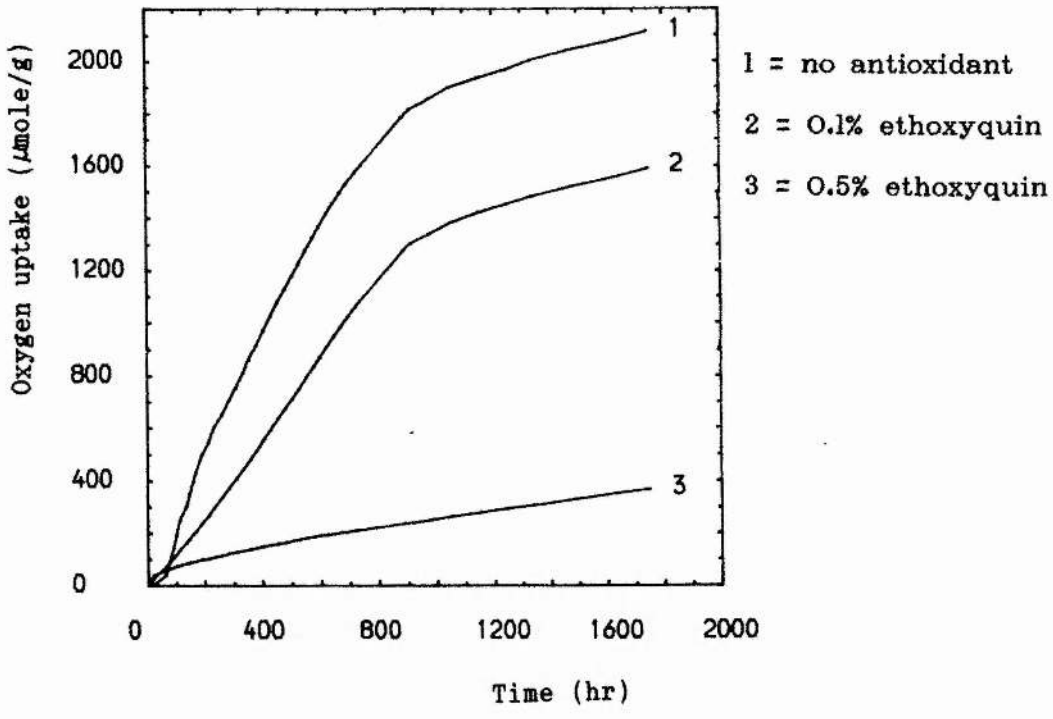


Figure 5.3.2 illustrates that ethoxyquin has strong antioxidant effects in the South African meal. After a short inhibition period (70 hours), the control has a constant rate of oxygen uptake of approximately 2.2 µmole/g hr and then the rate decreases. The meal containing 0.1% ethoxyquin takes up oxygen at a rate of 1.5 µmole/g hr initially, which then decreases. Apart from a short period in the beginning, the sample containing 0.5% ethoxyquin has a constant rate of oxygen uptake of 0.2 µmole/g hr throughout the experiment.

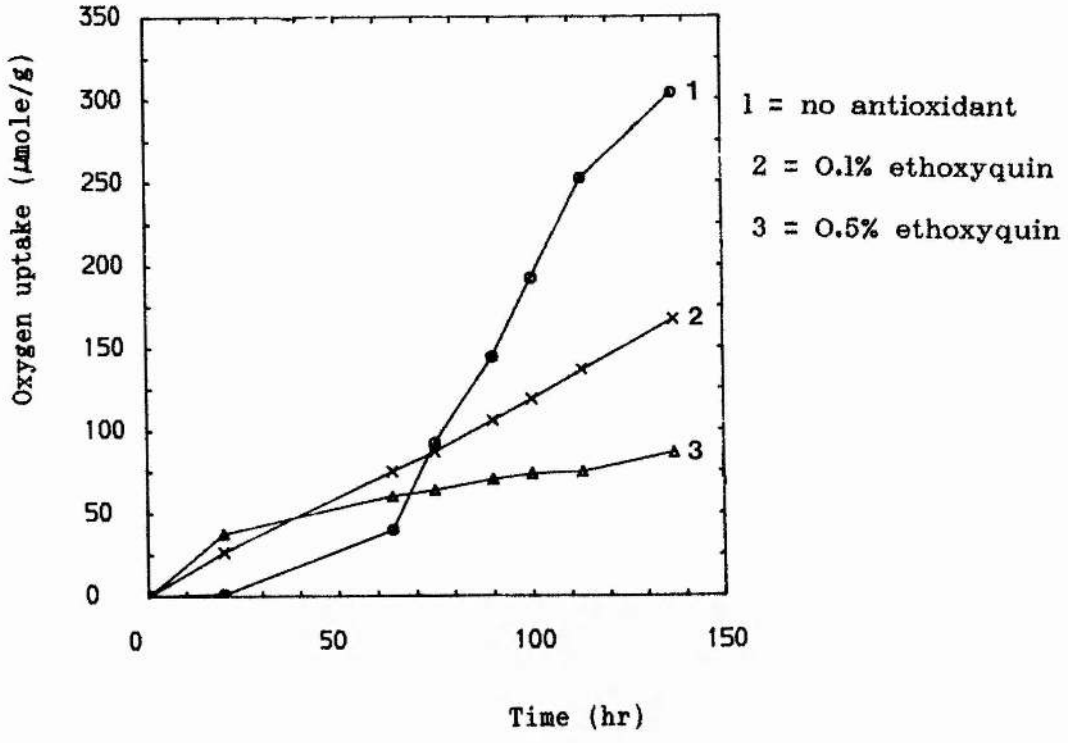
Figure 5.3.2 Autoxidation of South African fish meal (30 °C), oxygen uptake vs time.



As mentioned before, the fish meal containing no ethoxyquin had a short inhibition period, but when examined more closely (Figure 5.3.3) it becomes obvious that the samples containing ethoxyquin have an oxygen uptake which is faster to begin with but then decreases quickly. This is similar to the behaviour seen in some of the fish oils.

BHT proved to be a very inefficient antioxidant in this meal, with samples containing 0.1 and 0.5% BHT having an oxygen uptake rate of 2.0 and 1.3 µmole/g hr respectively.

Figure 5.3.3 Autoxidation of South African fish meal (30 °C), oxygen uptake vs time.

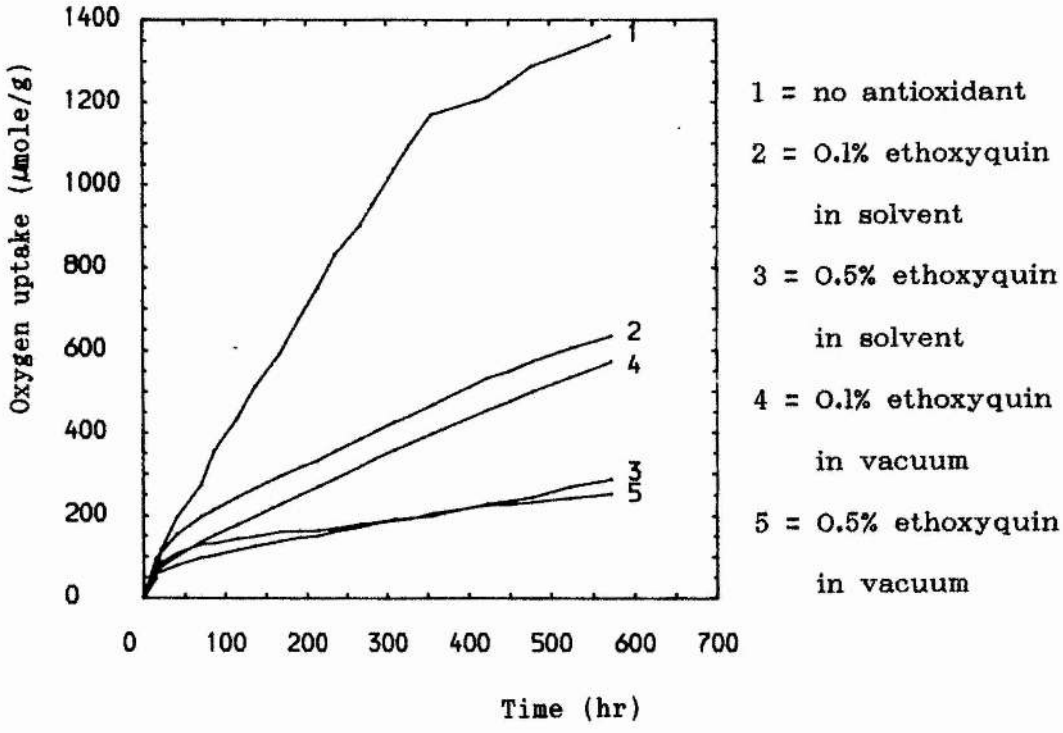


It has been shown previously that when ethoxyquin is added into fish meal the peroxide value drops (103, 104) whereas this value increases quickly for untreated meal. It is also known that the measureable amount of ethoxyquin in the meal initially decreases rapidly (105) and this might be due to a reaction of ethoxyquin with peroxides.

It is important, but difficult, to obtain a homogeneous mixing of ethoxyquin in the fish meal. In the previous experiments the antioxidant was added in a small amount of oil. Two other methods were tried, addition of ethoxyquin on the vapour phase under vacuum or dissolved in a large amount of hexane. There is not much

difference between oxygen uptake of meal treated either way (Figure 5.3.4), but the vacuum method seems to be slightly better. This could be because in the solvent method some of the lipids are extracted to the surface of the meal which makes them easier to autoxidise. It proved difficult to compare autoxidation experiments because the rate of oxygen uptake can vary considerably between experiments. When the controls in Figure 5.3.2 and 5.3.4 are compared, the initial oxygen uptake is higher in Figure 5.3.4. On the other hand, the methods used in that experiment to mix the ethoxyquin in the meal gave better results than the one in Figure 5.3.2

Figure 5.3.4 Autoxidation of South African meal (30 °C), oxygen uptake vs time.



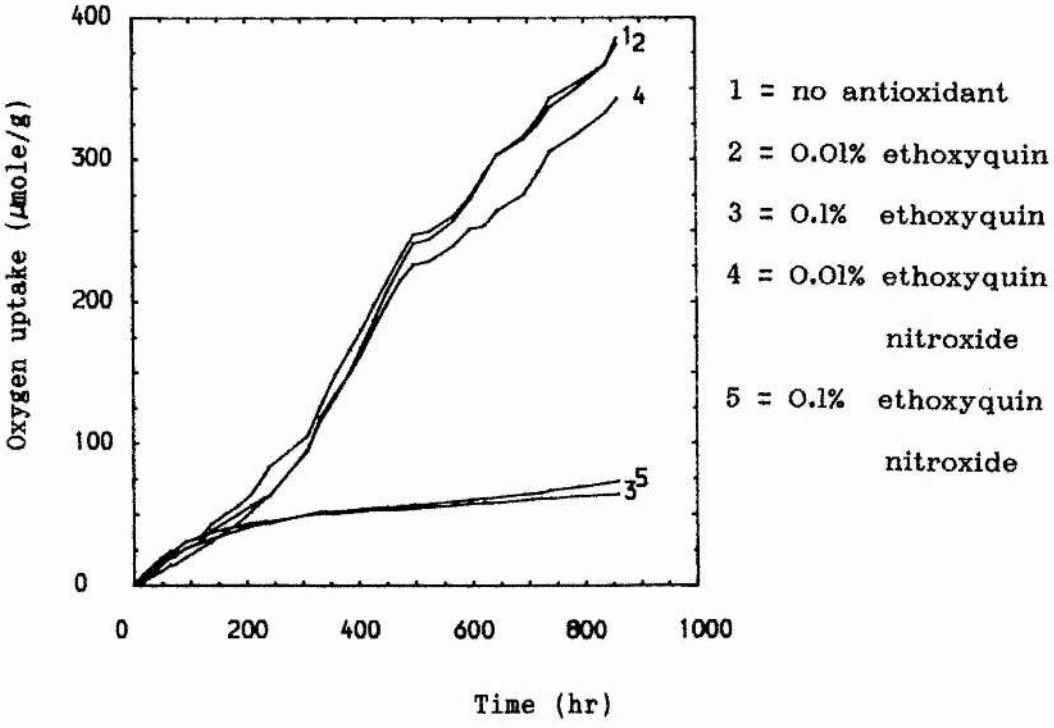
5.4 Ethoxyquin nitroxide in fish oils

It has been suggested that some amines, in particular secondary aromatic amines, owe some of their antioxidant properties to the formation of a nitroxide radical. These react with either alkyl or peroxy radicals of the autoxidising lipids (10, 18, 19, 106-108), but Brownlie and Ingold (22) concluded that nitroxides or their hydroxylamines were not likely to play an important role as commercial antioxidants because of their reduced efficiency and stability compared to the corresponding amines. Berger *et al* (18) showed that many alkyl-aryl and dialkyl nitroxides were more effective than diaryl nitroxides, most likely due to higher resistance to side reactions.

Ethoxyquin can be converted into its nitroxide, which can be isolated (sect 2.5), and Lin and Olcott (69) and Möhr (109) claimed that it was a better antioxidant than ethoxyquin.

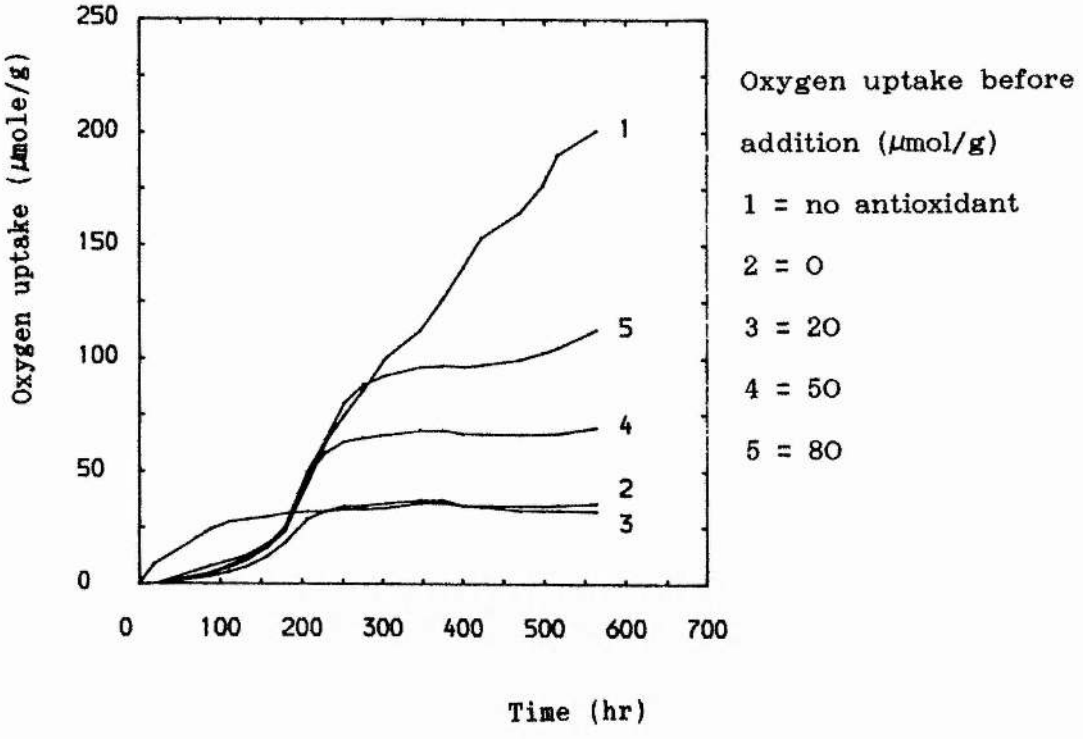
When ethoxyquin nitroxide was compared to ethoxyquin as an antioxidant for herring oil, the oxygen uptake was very much the same (Figure 5.4.1). At lower concentration (0.01%) neither compound has an antioxidant effect, but the samples containing the higher concentration (0.1%) behave as expcected for oils containing ethoxyquin. Slight pro-oxidant effects are seen initially and then the oxygen uptake decreases.

Figure 5.4.1 Autoxidation of herring oil (30 °C), oxygen uptake vs time.



Ethoxyquin nitroxide was also added to preoxidised mackerel oil, and when Figure 5.4.2 is compared to Figure 5.2.5 it can be seen that ethoxyquin stops the oxygen uptake faster and for a longer period. In fresh oil, ethoxyquin has better initial effects than the nitroxide which shows strong pro-oxidant effects when added to the fresh oil. It can also be seen that the initial oxidation level of the oil does not significantly alter the early shape of the oxygen uptake curves, whereas the antioxidant effects of ethoxyquin are more quickly observed in the preoxidised oil.

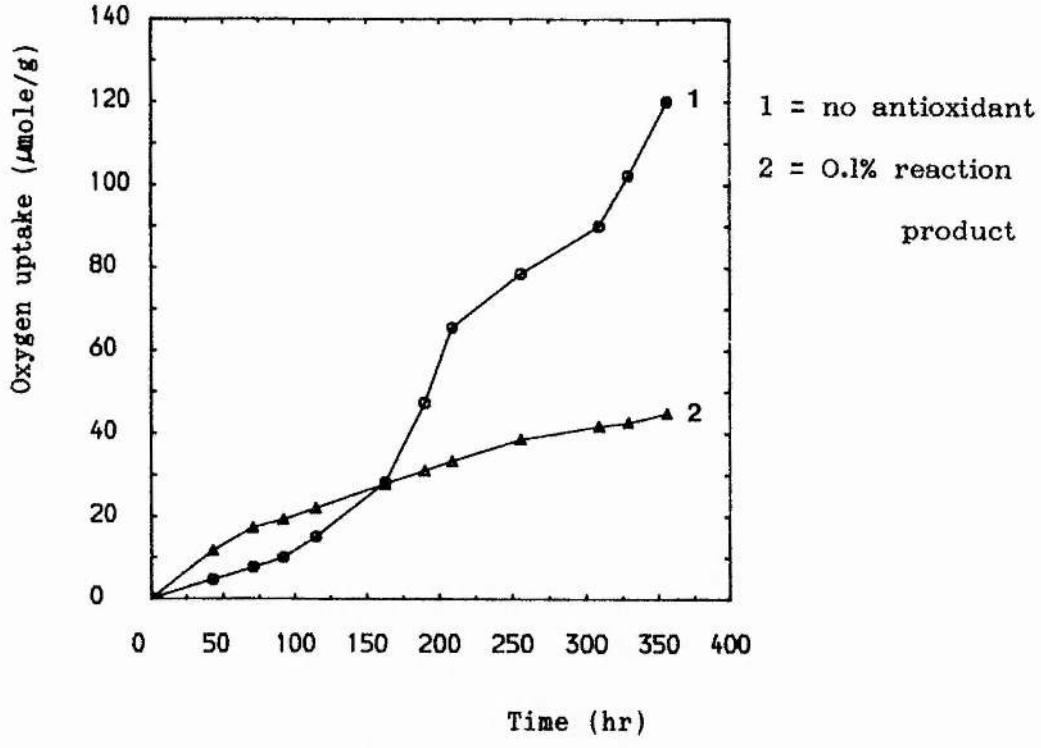
Figure 5.4.2 Autoxidation of mackerel oil (30 °C), ethoxyquin nitroxide (0.1%) added at different levels of oxygen uptake. Oxygen uptake vs time.



One can therefore conclude that ethoxyquin nitroxide is not an effective antioxidant in fish oil, but is converted to another more efficient antioxidant which is either formed in lower yield or is less effective than the products of ethoxyquin oxidation.

To see if the superior antioxidant effects reported previously (69, 109) were possibly due to some impurities present in the nitroxide, the crude reaction product (without chromatographic purification) was added to mackerel oil (Figure 5.4.3). This mixture seems to have effects similar to the pure nitroxide, that is a short period of pro-oxidant activity which is followed by a period of very low oxygen uptake.

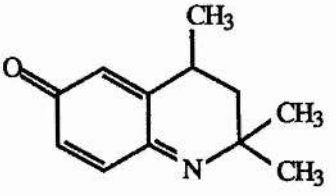
Figure 5.4.3 Autoxidation of mackerel oil (30 °C), oxygen uptake vs time.



To summarise, ethoxyquin nitroxide was made and tested to see if it could be the oxidation product of ethoxyquin that caused sudden decrease in oxygen uptake. Those experiments suggest that this is not the case.

5.5 Antioxidant properties of 2,6-dihydro-2,2,4-trimethyl-6-quinolone and 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline

Ethoxyquin oxidised by either tert-butylhydroperoxide in the presence of ammonium ferrous sulphate, or in oxidised methyl linoleate was shown to form 2,6-dihydro-2,2,4-trimethyl-6-quinolone. Quinone compounds are known to have antioxidant properties but mainly as alkyl-radical acceptors, which usually means that their antioxidant properties are best seen at low oxygen pressures (10, 106, 110). Kasaikina *et al* (110) found that 2,3,4,6-tetrahydro-2,2,4-trimethyl-6-quinolone (I) is an alkyl-radical acceptor, but it did not react with peroxy-radicals. On the other hand, Varlamov and Denisov (111) found that the quinone-imine 4-(phenylimino)cyclohexa-2,5,dien-1-one (II) probably reacts with both alkyl- and peroxy-radicals. They also found that during that process, reduction of the quinone-imine to its corresponding hydroxy-amine (III) occurred and that this is a very strong autoxidation inhibitor.



I

II III

Figure 5.5.1 shows the autoxidation of mackerel oil containing ethoxyquin or the 6-quinolone isolated from ethoxyquin after oxidation by tert-butylhydroperoxide or oxidised methyl linoleate. As has been seen before, the oil containing ethoxyquin has a rather fast initial oxygen uptake which then decreases. On the other hand, both the 6-quinolone samples gave almost no oxygen uptake throughout the experiment (1380 hours).

In the South African fish meal (Figures 5.5.2), there is very little if any difference between the antioxidant acitivity of ethoxyquin and the 6-quinolone.

For a quinone-like compound, 2,6-dihydro-2,2,4-trimethyl-6-quinolone has surprisingly good antioxidant properties.

Various hydroxyquinolines have been reported as good antioxidants. Bickoff *et al* (112) showed that in mineral oil, 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline gave the most powerful protection for carotene among 40 different quinoline and hydroxyquinoline derivatives. On the other hand, the 6-hydroxy compound was not so powerful in alfalfa meal, perhaps because of its poor solubility in the lipid phase. Figure 5.5.3 shows autoxidation of mackerel oil containing ethoxyquin or 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline. Ethoxyquin at 0.01% gives an inhibition period of 120 hours, but the same concentration of the 6-hydroxy derivative gives a period of 200 hours. There is no difference between samples containing 0.05 and 0.1% ethoxyquin, both have a fast initial oxygen uptake which then decreases. On the other hand, 0.1% of the 6-hydroxy derivative inhibits nearly all oxygen uptake throughout the experiment.

Figure 5.5.1 Autoxidation of mackerel oil (30 °C), oxygen uptake vs time.

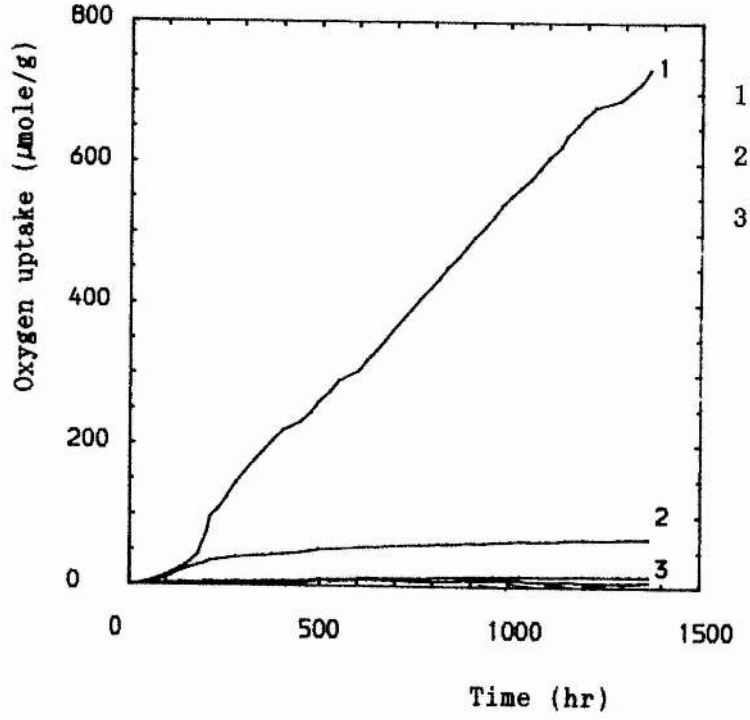


Figure 5.5.2 Autoxidation of South African fish meal (30 °C), oxygen uptake vs time.

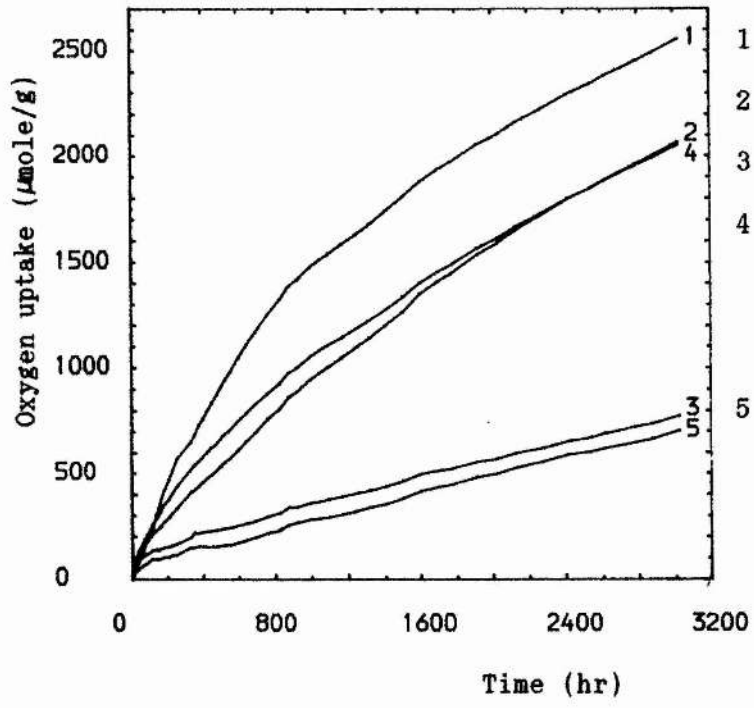
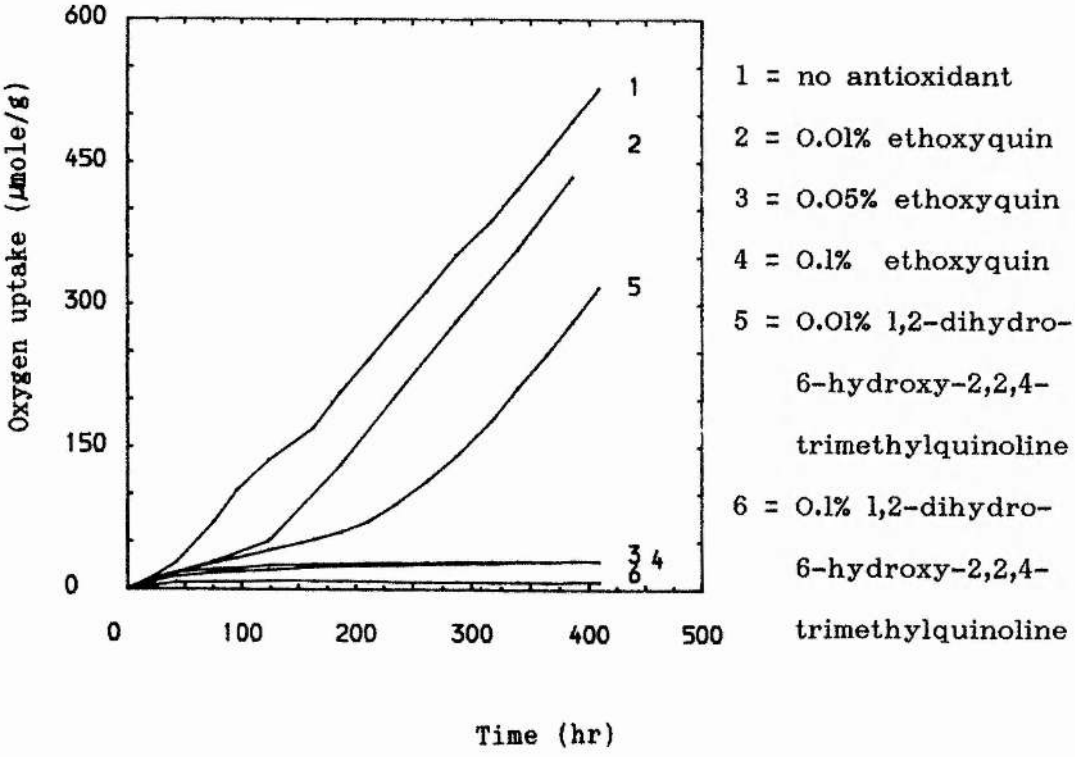


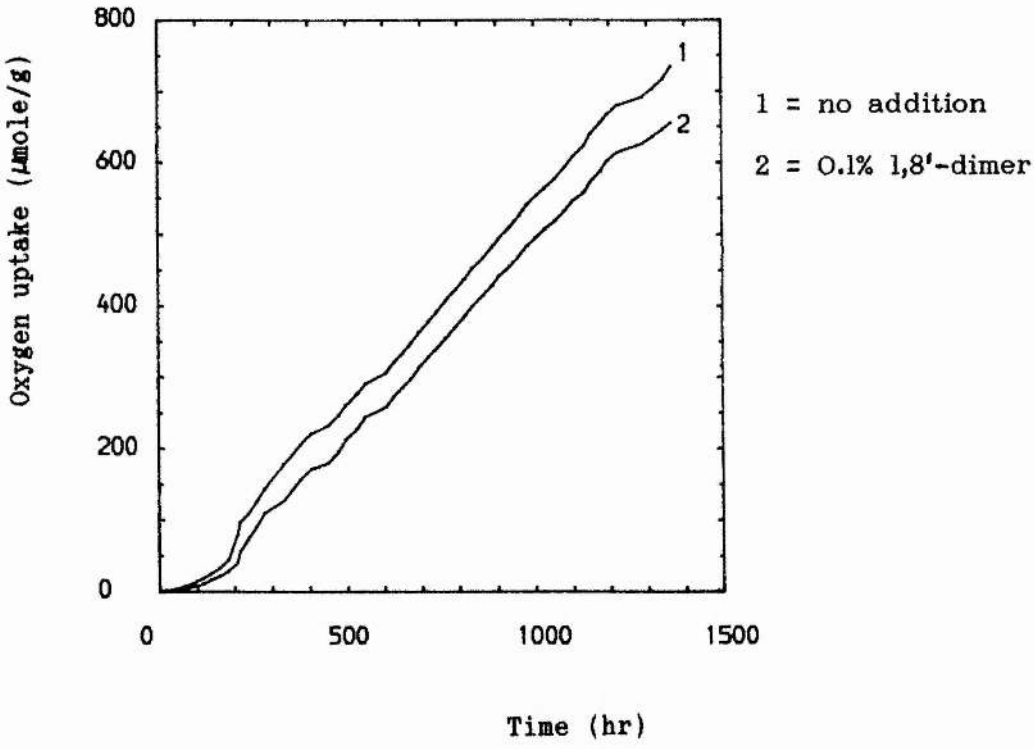
Figure 5.5.3 Autoxidation of mackerel oil (30 °C), oxygen uptake vs time.



5.6 Effects of the 1,8'-ethoxyquin dimer and 2,4-dimethyl-6-ethoxyquinoline on oil autoxidation

A major oxidation product of ethoxyquin is its 1,8'-dimer. This compound was added to mackerel oil to see if it had any antioxidant properties (Figure 5.6.1)

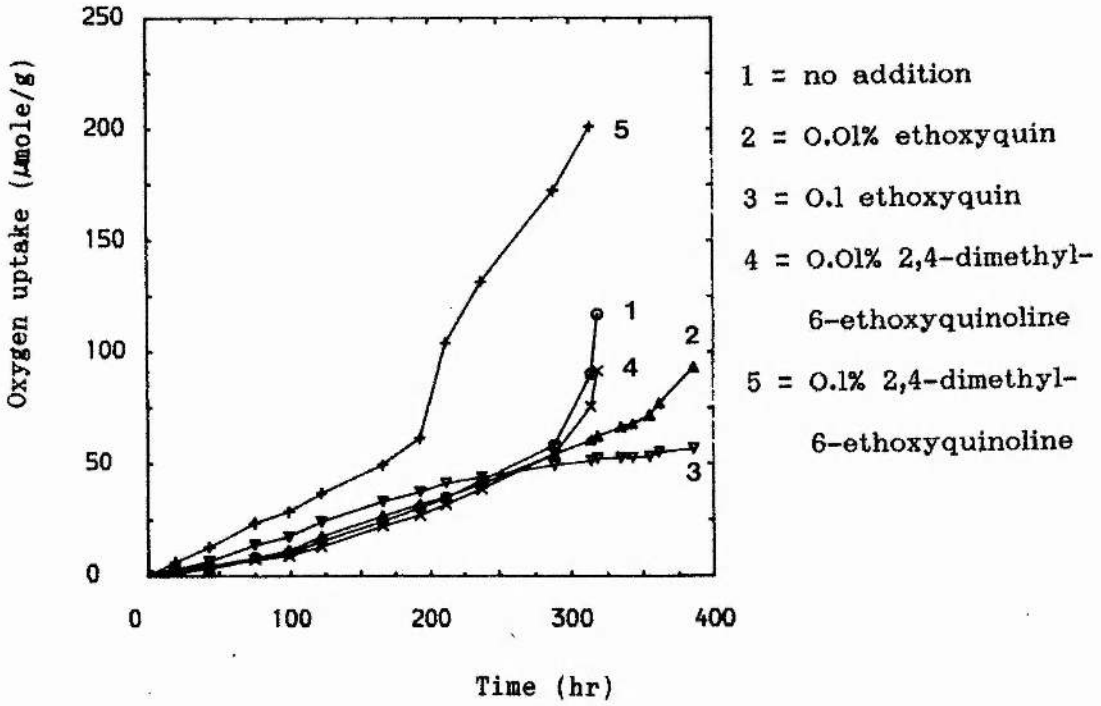
Figure 5.6.1 Autoxidation of mackerel oil (30 °C), oxygen uptake vs time.



The dimer has only one amino hydrogen and it is not easily approached with an ethoxyquin group in the 8-position. Therefore, as was expected, there is no significant difference between the oxygen uptake of the samples containing the dimer (0.1%) and the controls.

The 2,4-dimethyl-6-ethoxyquinoline, formed by reaction of ethoxyquin and oxygen at high temperature, was tested for antioxidant properties in cod liver oil (Figure 5.6.2).

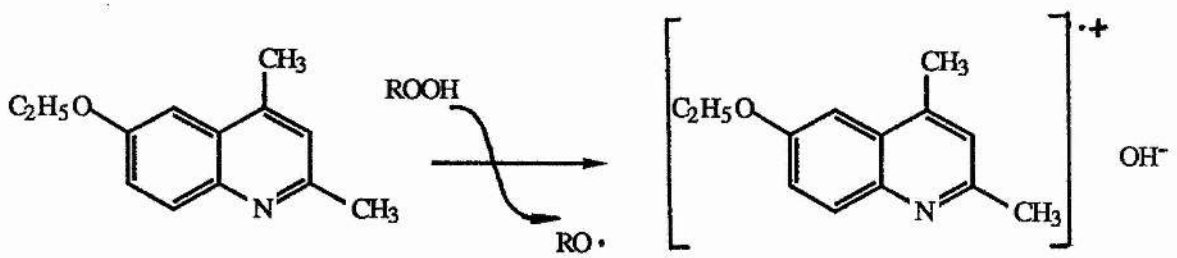
Figure 5.6.2 Autoxidation of cod liver oil (30 °C), oxygen uptake vs time.



The samples containing 0.01% of the dimethylquinoline have the same oxygen uptake as the controls, whereas 0.01% ethoxyquin gives a slight protection. On the other hand, where effects of 0.1% ethoxyquin are increasing towards the end of the experiment, the same concentration of the dimethylquinoline has strong pro-oxidant effects, reducing the inhibition period of the untreated oil from 300 hours to approximately 200 hours. According to Bickoff *et al* (112) this compound has no effects on carotene stabilisation. Adamic *et al* (20) have shown that 1,4-bis-dimethylaminobenzene can act like reducing transition metals such as Co^{2+} and Fe^{2+}. These break down hydroperoxides to form alkoxy-radicals which catalyse autoxidation.

$$Fe^{+2} + ROOH \longrightarrow (Fe^{+3})OH^{-} + RO\cdot$$

It is possible that 2,4-dimethyl-6-ethoxyquinoline behaves in the same way, that is:



CHAPTER 6

OXIDATION REACTIONS OF ETHOXYQUIN AND SOME OF ITS OXIDATION PRODUCTS

6.1 Introduction

The work described in this chapter was carried out to gain further understanding of the reactions of ethoxyquin and some of its oxidation products in autoxidising systems.

Results and Discussion

6.2 Oxidation reactions of ethoxyquin

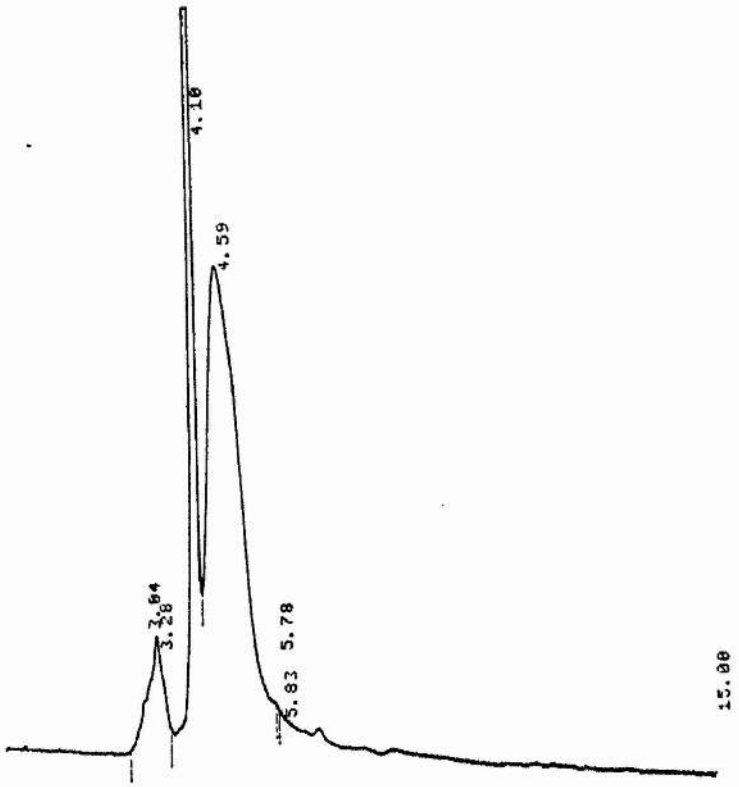
Petrescu (103, 104) suggested that peroxides already present in fish meal are attacked by added ethoxyquin with a consequent decrease in peroxide value and in the level of ethoxyquin. We observed no change in the UV-spectrum of an equimolar solution of ethoxyquin and tert-butylhydroperoxide in ethanol over 24 hours at room temperature, indicating that this hydroperoxide is not attacked by ethoxyquin. We have, however, already shown that tert-butoxy-radicals react readily with ethoxyquin. This suggests that ethoxyquin reacts with the radicals produced in the autoxidising system rather than with the hydroperoxides.

When ethoxyquin (1%) was added to autoxidising methyl linoleate (30 ^{o}C), the rate of oxygen uptake dropped immediately from 0.65 to 0.1 μmole/g hr. The two major oxidation products of ethoxyquin were the 1,8'-dimer and 2,6-dihydro-2,2,4-trimethyl-6-quinolone which could be extracted with acid and purified for analysis by preparative HPLC. The dimer could not be extracted from a hexane solution with acid and was only identified by TLC as a fluorescent spot overlapping the linoleate.

It was shown that the 6-quinolone compound was formed in fish meal containing ethoxyquin (0.5%), which had been autoxidised for

one week, by HPLC of an acid extract from the lipid phase. By use of standards, the acid extract was found to contain ethoxyquin (88%) and 2,6-dihydro-2,2,4-trimethyl-6-quinolone (12%). The formation of the dimer was demonstrated by use of HPLC of the lipid phase from meal after autoxidation for 60 days (Figure 6.2.1).

Figure 6.2.1 HPLC chromatogram of fish meal lipid phase.

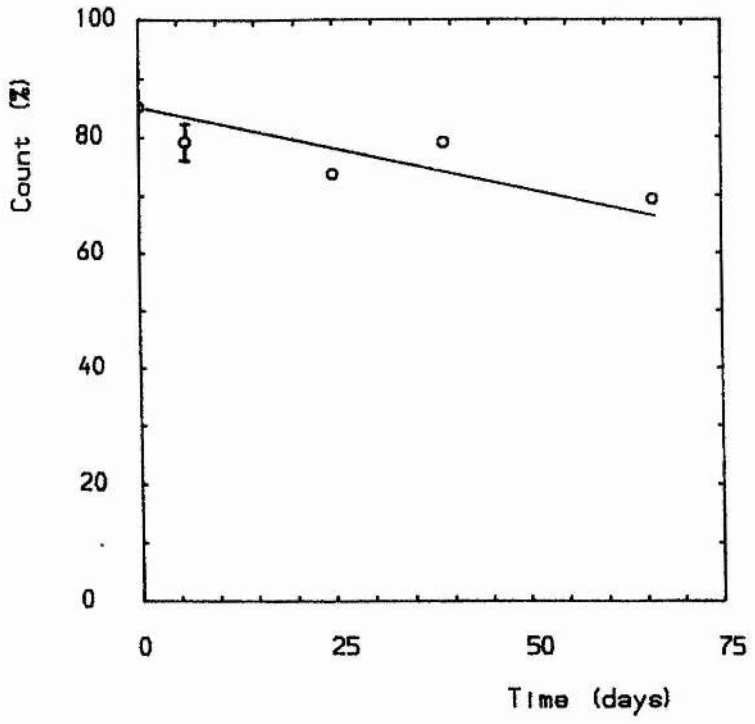


By use of a Polychrom detector, the peak at 4.1 minutes was shown to have the same UV-spectrum as the 1,8'-dimer which is also eluted at 4.1 minutes.

Figure 6.2.2 illustrates the slight decrease in radiolabelled material extractable from fish meal (Total extract). This indicates a possible reaction between ethoxyquin or its oxidation products with,

for example, the protein.

Figure 6.2.2 Total radiolabelled material extractable from fish meal, counts (%) vs time (days).



During the first 25 days the radioactive material bound to the non-extractable matrix (Figure 6.2.3) increases from less than 2% to approximately 14%, with little change thereafter. The error for the duplicates was in all cases less than 1% of total count.

The difference between the count in the total extract and the acid extractables should mainly represent the dimer (Figure 6.2.4) and is approximately 11% throughout the experiment. It is not surprising that most of the dimer is formed in the early stages of the reaction, when the concentration of ethoxyquin is highest.

Figure 6.2.3 Non-extractable radiolabelled material, counts (%) vs time (days).

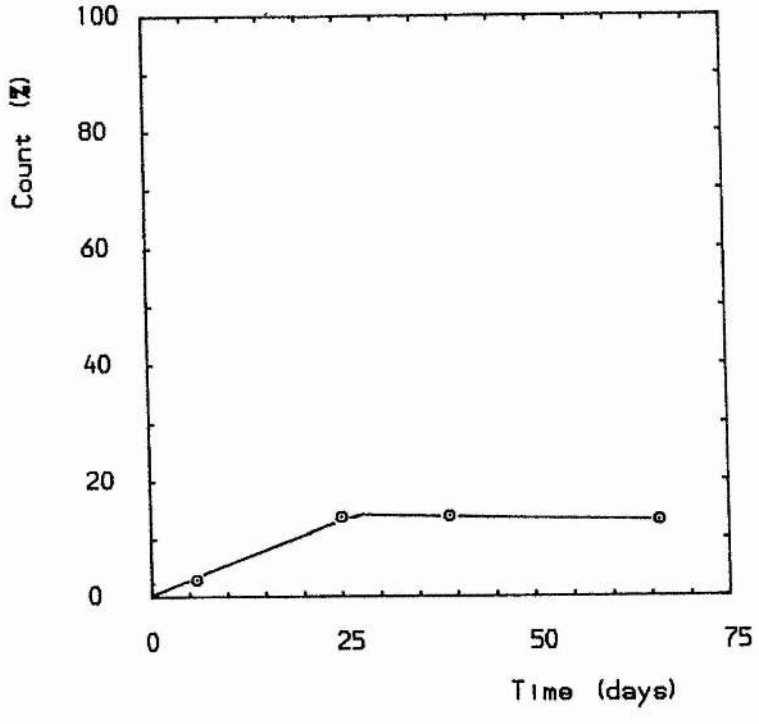
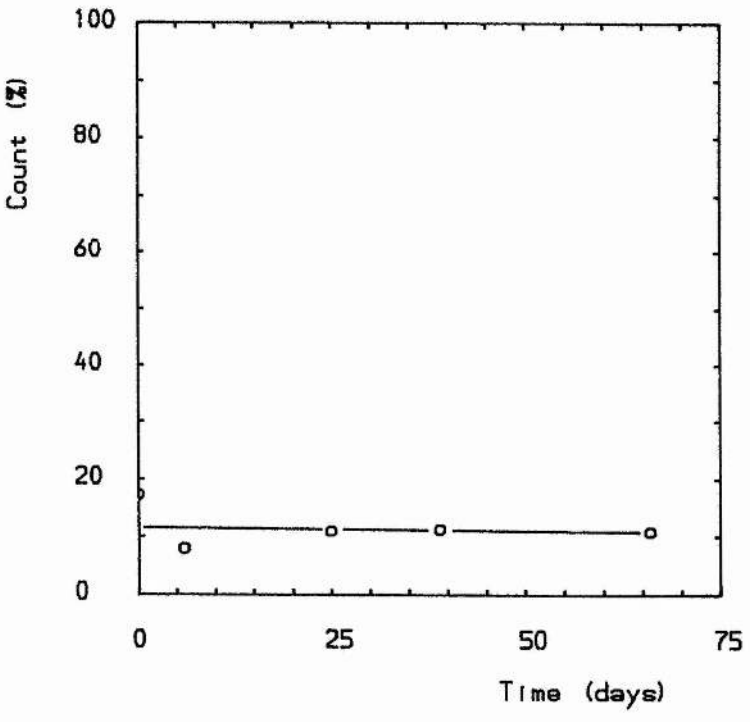


Figure 6.2.4 Non-acid extractable radiolabelled material, counts (%) vs time (days).



Figures 6.2.3 and 6.2.4 illustrate that 25 days after addition, approximately 25% of the ethoxyquin is either non-extractable or is in the dimer form. Figure 6.2.5 shows the counts in the second hexane extract which represents the acid soluble material, mainly ethoxyquin and 2,6-dihydro-2,2,4-trimethyl-6-quinolone. Unfortunately something went wrong in the first two measurements, but if they are excluded it can be seen that after 25 days only about 30% of added ethoxyquin is extracted by acid and it changes little between 25 and 65 days.

Figure 6.2.5 Acid extractable radiolabelled material, counts (%) vs time (days).

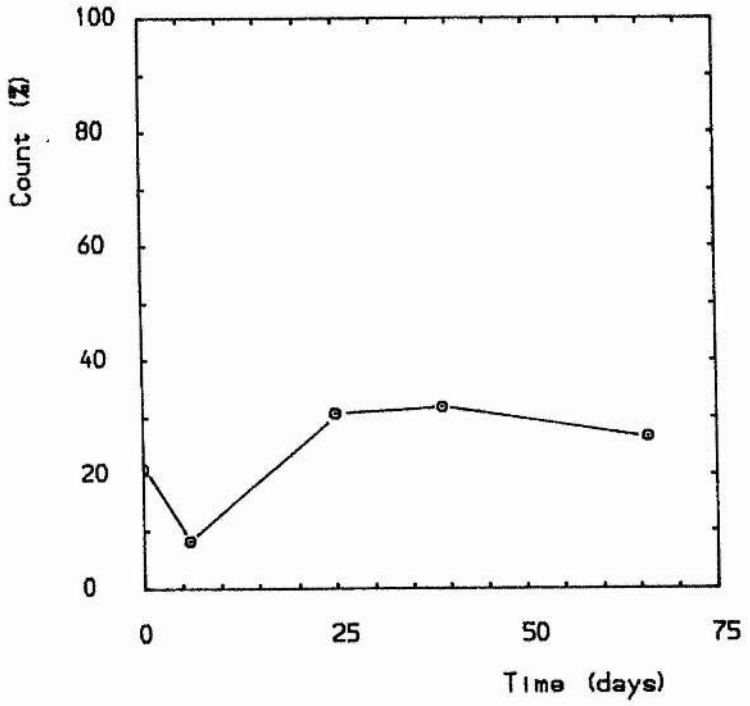
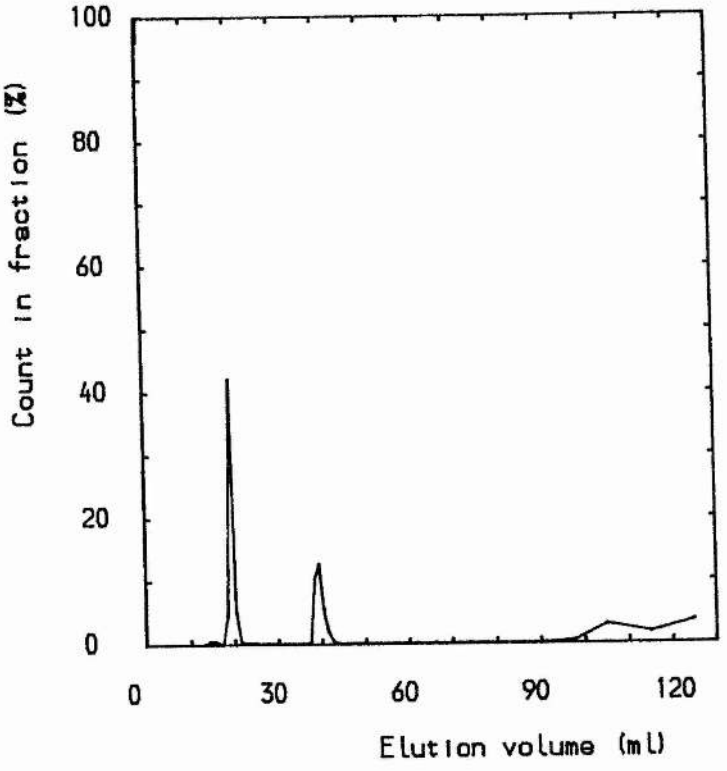


Figure 6.2.6 shows results from a typical preparative HPLC run for the acid extractable material where the counts in each fraction are plotted against elution volume. Ethoxyquin is eluted between 18 and 25 ml and the 6-quinolone between 36 and 45 ml.

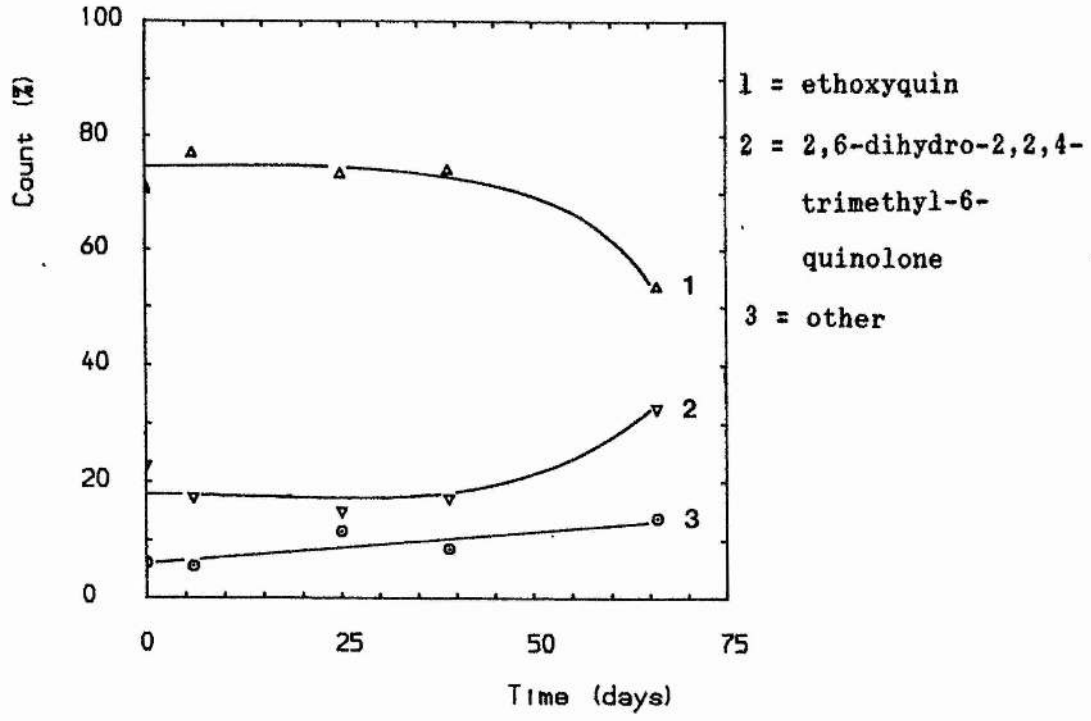
Figure 6.2.6 Fractions from preparative HPLC run of acid extractable radiolabelled material, counts (%) vs time (days).



In Figure 6.2.7, the results from the preparative HPLC runs over the experimental period are expressed as percentage of each compound in the acid extract. The ethoxyquin is initially approximately 75% but decreases to 45%, whereas 2,6-dihydro-2,2,4-trimethyl-6-quinolone is increasing from 15 to 33%. Therefore, when

levels of antioxidants in the meal are estimated, it is very important to measure 2,6-dihydro-2,2,4-trimethyl-6-quinolone as well. There is no indication of any other compounds formed in significant amounts apart from the 1,8'-dimer.

Figure 6.2.7 Composition of radiolabelled acid extract, counts (%) vs time (days).



Contreias (113) obtains between 70 and 80% recoveries straight after addition of ethoxyquin to fish meal and less than 50% after two months. Wessels *et al* (105) showed that various fish meals can lose more than 50% of added ethoxyquin in about 20 days, but Spark (28) got over 90% recovery by the use of the same method. The methods

mentioned above are based on either colorimetric determination at 362 nm, or by reaction of the antioxidant with 1,1-diphenyl-2-picrylhydrazyl and determination of decrease in absorbance at 517 nm. It is doubtful whether these methods are specific for ethoxyquin and therefore probably lead to overestimation. Dahle and Skaare (114) used a gas chromatographic method to specifically measure ethoxyquin and only obtained 30% recovery from various systems.

6.3 GC-MS studies of reactions of ethoxyquin and some of its oxidation products in fish oil

Tables 6.3.1 - 6.3.4 list the main ions from the major components extracted from oil after additon of ethoxyquin and some of its oxidation products.

Table 6.3.1 GC-MS data for EQ-oil reaction product.

Compound (identity)	retention (sec)	Fragment m/e (relative abundance)					
I (QLO)	269	187 (93)	172 (27)	159 (45)	144 (100)		
II (EQ)	299	217 (37)	202 (100)	188 (13)	174 (100)	159 (10)	145 (51)
III (QL)	310	201 (63)	173 (100)	158 (4)	144 (18)		

QLO = 2,6-dihydro-2,2,4-trimethyl-6-quinolone
EQ = ethoxyquin
QL = 2,4-dimethyl-6-ethoxyquinoline

Apart from II (EQ) two products are seen. The first peak I (QLO) has a typical mass spectrum for 2,6-dihydro-2,2,4-trimethyl-6-quinolone and the last one III (QL) for 2,4-dimethyl-6-ethoxy-quinoline. This quinoline derivative is formed only in very small amounts as a product of low temperature oxidation of ethoxyquin, but it is known to be formed when pure ethoxyquin is examined on the same GLC system, in different amounts depending on the injection temperature.

No changes were observed on 2,4-dimethyl-6-ethoxyquinoline when it was added to autoxidising oil.

Table 6.3.2 shows the GC-MS data for the ethoxyquin nitroxide (EQN) reaction products.

Compund I (QLO) and II (EQ) are readily identified as 2,6-dihydro-2,2,4-trimethyl-6-quinolone, which is probably formed through its N-oxide and ethoxyquin. Two compounds have a molecular ion 232 (IV and V) but neither has exactly the same mass spectrum as the pure nitroxide. Two other peaks are shown here, with possible molecular ions 223 (VI) and 252 (VII) but they have not been identified.

Table 6.3.2 GC-MS data for EQN-oil reaction product.

Compound (identity)	Retention (sec)	Fragment m/e (relative abundance)						
I (QLO)	268	187 (100)	172 (30)	159 (50)	144 (90)			
II (EQ)	301	217 (23)	202 (100)	188 (6)	174 (70)	145 (23)		
IV	304	232 (13)	216 (70)	202 (100)	188 (21)	187 (13)	174 (15)	
		173 (96)	158 (12)	145 (30)	144 (30			
V	312	232 (11)	216 (100)	201 (5)	188 (15)	173 (25)	159 (11)	
VI	323	223 (47)	219 (6)	205 (15)	204 (14)	176 (6)	167 (18)	149 (100)
VII	334	252 (18)	234 (80)	218 (77)	206 (100)	204 (79)	191 (27)	
		188 (31)	176 (28)	160 (33)	148 (18)	146 (19)	134 (16)	132 (14)

EQN = ethoxyquin nitroxide

In Table 6.3.3 the GC-MS data for reaction products of 2,6-dihydro-2,2,4-trimethyl-6-quinolone-N-oxide (QLO-N-OX) is shown

Table 6.3.3 GC-MS data for QLO-N-OX-oil reaction product.

Compound (identity)	Retention (sec)	Fragment m/e (relative abundance)							
I (QLO)	270	187 (96)	172 (28)	159 (45)	144 (100)				
VII (QLO-N-OX)	340	203 (100)	188 (34)	186 (44)	173 (11)	171 (10)	160 (13)	158 (25)	145 (49)

The main product of the N-oxide (VII) is the corresponding quinolone (I). It is not known why the mass spectrum of the N-oxide is not as has been seen before (section 2.5).

Finally, Table 6.3.4 shows the GC-MS data for reaction products of 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline (HQL).

Table 6.3.4 GC-MS data for HQL-oil reaction product

Compound (identity)	Retention (sec)	Fragment m/e (Relative abundance)			
I (QLO)	272	187	172	159	144

The only reaction product of 1,2-dihydro-6-hydroxy-2-2,4-trimethylquinoline (HQL) is the quinolone (I). This reaction occurs very readily and in fact when the pure compound is injected it is all converted on the GC system.

To summarise, ethoxyquin is oxidised to two major products, a 1,8'-dimer and 2,6-dihydro-2,2,4-trimethyl-6-quinolone. There is no indication that the 6-quinolone is formed *via* the corresponding hydroxyamine (1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline). Ethoxyquin is regenerated from its nitroxide but that could occur during the acid extraction, as the nitroxide is not stable in acidic conditions. The quinolone compound is also formed from the nitroxide *via* its N-oxide or ethoxyquin. There is on the other hand no indication of the nitroxide being formed in any significant amounts from ethoxyquin. 2,4-Dimethyl-6-ethoxyquinoline is seen in the ethoxyquin reaction mixture, but it is formed mainly on the GC-system rather than as a low temperature oxidation product.

CHAPTER 7

INHIBITION OF AIBN INITIATED AUTOXIDATION OF METHYL LINOLEATE BY ETHOXYQUIN AND THREE OF ITS OXIDATION PRODUCTS

7.1 Introduction

The autoxidation chain-reactions involve three steps, initiation, propagation and termination.

Initiation

$$RH \xrightarrow{k_i} R\cdot \qquad \text{Rate} = R_i$$

Propagation

$$R\cdot + O_2 \xrightarrow{k_o} ROO\cdot$$

$$ROO\cdot + RH \xrightarrow{k_p} ROOH + R\cdot$$

Termination

$$2ROO\cdot \xrightarrow{k_t}$$

$$ROO\cdot + R\cdot \xrightarrow{k'_t}$$

$$2R\cdot \xrightarrow{k''_t}$$

} Non-radical products

At steady state, it is assumed that the concentration of R· and ROO· does not change. If the partial pressure of oxygen is high, the termination steps involving R· can be ignored ($k_t >> k'_t + k''_t$) and therefore

$$\frac{d\,[R\cdot]}{dt} = R_i - k_o[R\cdot][O_2] + k_p[ROO\cdot][RH] = 0$$

and

$$\frac{d\,[ROO\cdot]}{dt} = k_o[R\cdot][O_2] - k_p[ROO\cdot][RH] - 2\,k_t\,[ROO\cdot]^2 = 0$$

By substitution

$$[RO_2\cdot] = \frac{(R_i)^{1/2}}{(2k_t)^{1/2}}$$

Then the build-up of hydroperoxides, or the overall oxidation rate can be described as

$$\frac{-d\,[O_2]}{dt} = \frac{d\,[ROOH]}{dt} = k_p[RH]\,\frac{(R_i)^{½}}{(2k_t)^{½}} \qquad (I)$$

By assuming at low oxygen partial pressure that

$$(k_t')^2 = k_t \times k_t''$$

the rate of oxidation can be expressed as

$$\frac{-d\,[O_2]}{dt} = \frac{k_p\,(R_i)^{½}\,[RH]}{(2k_t)^{½}} \times \frac{[O_2]}{[O_2] + \dfrac{(k_t'')^{½}\,k_p\,[RH]}{(k_t)^{½}\,k_o}}$$

which, at a very high substrate concentration and a constant rate of initiation, can be simplified to

$$\frac{-d\,[O_2]}{dt} = A \times \frac{[O_2]}{[O_2] + B}$$

This clearly shows that as the concentration (partial pressure) of oxygen increases, B becomes less important and the oxidation approaches a constant rate A.

Going back to reaction *I* (high oxygen partial pressure), R_i can be replaced by different terms, depending on the initiation conditions. In the monomolecular period of hydroperoxide decomposition, the initiation can be written as a metal catalysed

decomposition of hydroperoxides. Then

$$R_i = k_i [ROOH] [M]$$

where k_i is the monomolecular rate constant and M the metal. Therefore reaction *I* becomes

$$\frac{-d [O_2]}{dt} = \frac{k_p [RH] k_i^{1/2}}{(2k_t)^{1/2}} [ROOH]^{1/2} [M]^{1/2} \qquad (II)$$

In the bimolecular period,

$$R_i = k_i' [ROOH]^2 [M]$$

where k_i' is the bimolecular rate constant.

$$\frac{-d [O_2]}{dt} = \frac{k_p [RH] k_i'^{1/2}}{(2k_t)^{1/2}} [ROOH] [M]^{1/2} \qquad (III)$$

(7, 115, 116)

When a radical initiator (I_2) such as AIBN is used, the rate can be described by equation *I* where

$$I_2 \xrightarrow{k_d} 2\ I\cdot \qquad R_i = e\ 2\ k_d [I_2]$$

and e is the initiation efficiency and k_d the dissociation constant for the initiator (117).

In the presence of a chain-breaking antioxidant (AH), the termination steps can be written as

$$ROO\cdot + AH \xrightarrow{k_a} ROOH + A\cdot$$

and

$$(n - 1)\ ROO\cdot + A\cdot \longrightarrow \text{non-radical products}$$

where n is the stoichiometric factor for the antioxidant. Then the rate of oxidation is described by

$$\frac{-d\,[O_2]}{d_t} = \frac{k_p\,[RH]\ e\ 2\ k_d\,[I_2]}{n\ k_a\,[AH]} \qquad (IV)$$

The stoichiometric factor for an antioxidant can be determined by using the induction period method, where

$$n = \frac{R_i \times \tau}{[AH]_o}$$

τ is the induction period and $[AH]_o$ the initial antioxidant concentration (15, 118). The induction period is the time determined by the point of intersection of the tangents to the initial inhibited and final uninhibited rates of oxidation (15).

R_i is determined by using an antioxidant with a known stoichiometric factor, in our case BHT (n = 2) which has been recommended as a standard to measure stoichiometric factors for other antioxidants (15).

The initiator AIBN (2,2'-bis(isobutyronitrile)) decomposes at a constant rate to give two radicals.

$$I_2N_2 \xrightarrow{k_d} 2\ I\cdot + N_2$$

$$O_2 + I\cdot \longrightarrow IOO\cdot$$

$$RH + IOO\cdot \longrightarrow IOOH + R\cdot$$

The dissociation constant has been determined by various methods to give

$$k_d(Ms^{-1}) = 1.58 \times 10^{15}\ e^{-30.8/RT} \qquad (119)$$

and the initiation efficiency of each radical is given by

$$e = \frac{R_i}{2\ k_d\ [In]} \qquad (117)$$

In this chapter, the kinetics of the inhibition by ethoxyquin and three of its oxidation products in AIBN initiated autoxidation of methyl linoleate is examined.

7.2 Results and discussion

Figure 7.2.1 shows the AIBN initiated oxygen uptake for methyl linoleate at 50 ^{o}C and in Table 7.2.1, the values used for the calculations of the initiation rate (R_i) are shown. The initiation rate was calculated by the induction period method using BHT as an inhibitor.

Figure 7.2.1 Oxygen uptake of methyl linoleate in chlorobenzene (6.8 x 10^{-1} M) at 50 °C. AIBN (4.5 x 10^{-2} M).

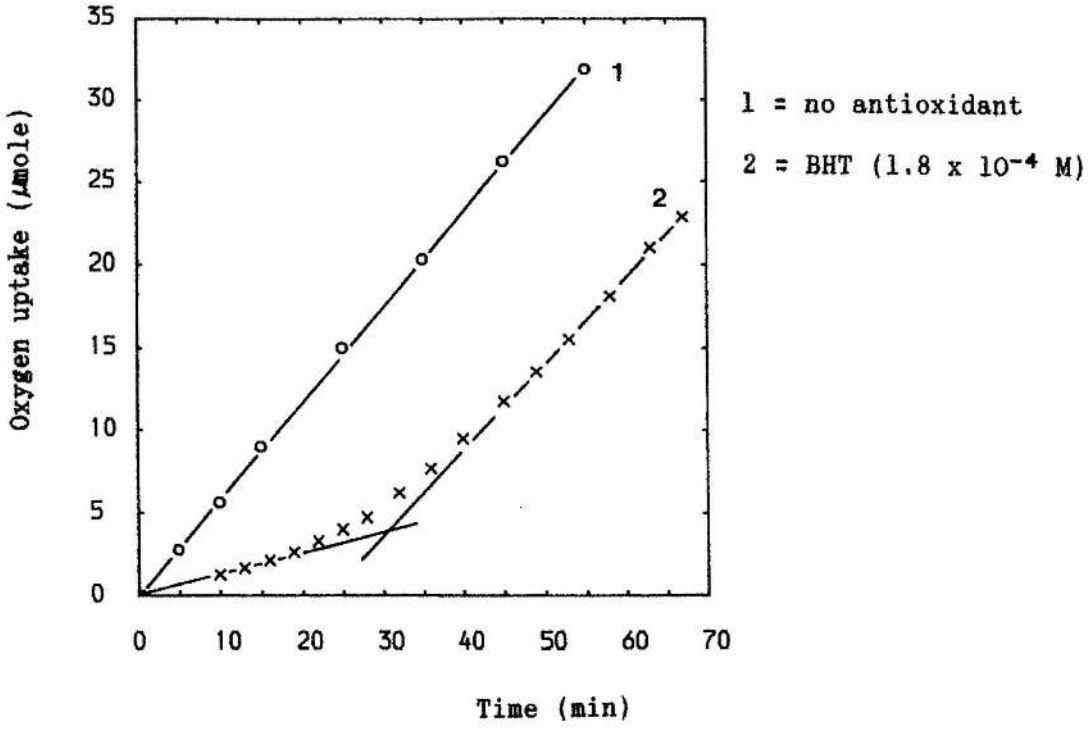


Table 7.2.1 Initiation rate (R_i) at 50 °C

BHT (M x 10^4)	τ^a (min)	R_i (Ms^{-1} x 10^7)
1.1	20.0	1.8
1.1	20.0	1.8
1.8	31.5	1.9
1.8	34.0	1.8
3.6	72.0	1.7
3.6	57.0	2.1
	Mean (s)	1.8 (0.15)

[a] τ is induction period

The mean initiation rate 1.8 x 10^{-7} Ms^{-1} will be used in all calculations. The calculated efficiency is 91% which is quite high

compared to values published for various other systems (15, 120). The same efficiency was assumed for oxidation at 30 °C.

Table 7.2.2 shows the results obtained for uninhibited linoleate oxidation. The term $k_p(2k_t)^{-½}$ which is obtained from equation *I*,

$$\frac{k_p}{(2k_t)^{½}} = \frac{-d\,[O_2]}{dt} \times \frac{1}{[RH]\,R_i^{½}}$$

has been described as the oxidisability of the substrate (117). The chainlength (ν) is calculated from

$$\nu = \frac{-d\,[O_2]}{dt} \times \frac{1}{R_i}$$

The oxidisability compares well with previously published results for methyl and ethyl linoleate under various conditions (2.0 - 2.8 x 10^{-2} $M^{-½}s^{-½}$) which indicates that the classical kinetic law is followed (117, 121-123). This is also supported by the very long chain length.

Table 7.2.2 Initiated oxidation of methyl linoleate in chlorobenzene (6.8×10^{-1} M) at 50 °C. AIBN (4.5×10^{-2} M)

Temp (°C)	$k_p(2k_t)^{-½}$ ($M^{-½}s^{-½} \times 10^2$)	ν
30	2.5	183.0
30	2.5	186.1
Mean (s)	2.5	184.6 (2.2)
50	2.5	40.7
50	2.6	42.1
50	2.3	37.3
50	2.3	36.6
	2.4 (0.2)	39.2 (2.6)

In Table 7.2.3 the results from use of various antioxidants are shown. The term $k_p k_a^{-1}$ is calculated from the integrated form of equation *IV*

$$[O_2]_{t=0} - [O_2]_t = - \left[\frac{k_p\,[RH]}{k_a}\right] \ln\,(1 - t/\tau)$$

and the chain length from

$$\nu = \frac{[O_2]_t - [O_2]_{t=0}}{n\,[AH]_o\,(1 - t/\tau)\,\ln\,(1 - t/\tau)} \qquad (15)$$

Both $k_p k_a^{-1}$ and ν were calculated at two points for each run (t/τ = 0.5 and 0.75) and the oxygen uptake was extrapolated to t = 0.

Table 7.2.3 Initiated and inhibited oxidation of methyl linoleate in chlorobenzene (6.8 x 10^{-1} M) at 50 °C. AIBN (4.5 x 10^{-2} M)

Antioxidant	c (M x 10^4)	τ (min)	t/τ	ν	$k_p k_a^{-1}$ (x10^3)	n
[b]BHT	1.1	20.0				2.0[a]
[b]BHT	1.1	20.0				2.0
BHT	1.8	31.5	0.5	13.1	3.5	2.0
			0.75	22.9	3.1	
BHT	1.8	34.0	0.5	11.9	3.2	2.0
			0.75	20.1	2.7	
BHT	3.6	72.0	0.5	6.2	3.3	2.0
			0.75	12.5	3.3	
BHT	3.6	57.0	0.5	8.0	4.2	2.0
			0.75	13.5	3.5	
Average (s)					3.4(0.4)	
EQ	3.2	79.5	0.5	1.3	0.7	2.7
			0.75	1.9	0.5	
EQ	3.2	59.0	0.5	1.0	0.7	2.0
			0.75	1.6	0.6	
EQ	4.8	101.0	0.5	0.6	0.5	2.3
			0.75	0.9	0.4	
EQ	4.8	120.0	0.5	0.9	0.7	2.7
			0.75	1.7	0.6	
EQ	6.4	116.0	0.5	2.1	2.2	2.0
			0.75	3.1	1.7	
EQ	6.4	107.0	0.5	2.7	2.9	1.8
			0.75	4.0	2.1	
EQ	9.6	242.0	0.5	1.2	1.9	2.7
			0.75	1.7	1.5	
EQ	9.6	222.0	0.5	1.1	1.7	2.5
			0.75	1.6	1.3	
Average (s)					1.3(0.8)	2.3(0.4)
EQN	2.2	41.0	0.5	4.1	1.3	2.0
			0.75	9.4	1.4	
EQN	2.2	39.0	0.5	2.9	0.9	1.9
			0.75	4.6	0.7	
EQN	4.4	77.0	0.5	6.8	4.1	1.9
			0.75	10.8	3.3	
EQN	4.4	68.0	0.5	4.3	2.6	1.7
			0.75	7.1	2.2	
Average (s)					2.1(1.2)	1.9(0.1)
[c] HQL	1.3	33.5				2.7
HQL	1.3	32.0				2.6
HQL	2.7	57.0				2.3
HQL	2.7	62.0				2.5
Average (s)						2.5(0.2)

BHT - butylated hydroxytoluene

EQ - ethoxyquin

EQN - ethoxyquin nitroxide

HQL - 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline

a literature value

b inhibition period was too short for accurate estimation of the inhibited oxygen uptake

c no oxygen uptake was observed during inhibition period

Ethoxyquin has a stoichiometric factor of 2.3, but the standard deviation is quite high. However, if the values for the 6.4×10^{-4} M concentration where the induction period was relatively much shorter are excluded, the stoichiometric factor becomes 2.5 (0.3). The $k_p k_a^{-1}$ value is noticeably lower for ethoxyquin than BHT which indicates a faster termination. Furthermore, the chain length for similar concentrations is much longer for the BHT inhibited oxidations.

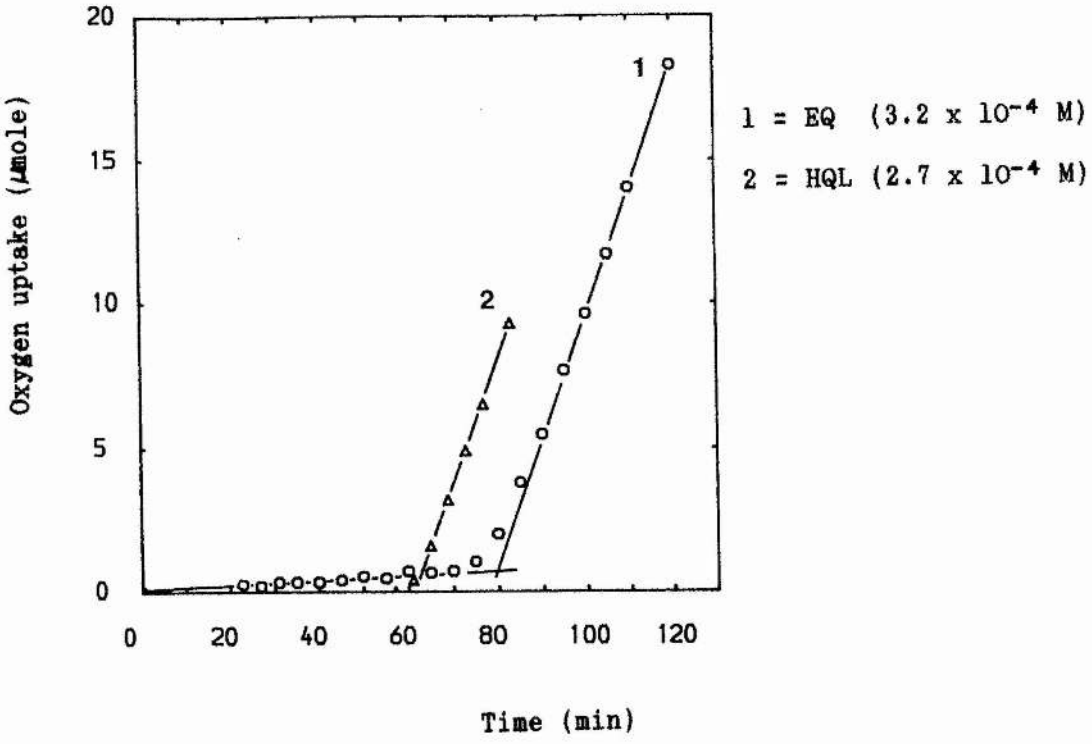
Although not a hydrogen donating antioxidant, the nitroxide has slightly higher termination rate constant (lower $k_p k_a^{-1}$) than BHT. The chain length is also shorter but the stoichiometric factor is only 1.9 (0.1).

The hydroxyquinoline is a very good hydrogen donor as can be seen from the fact that under the experimental conditions employed, no oxygen uptake was observed during the inhibition period. Therefore, estimation of ν and $k_p k_a^{-1}$ was impossible. The stoichiometric factor was calculated to be 2.5 (0.2) which is similar to that for ethoxyquin.

According to Burton and Ingold (15), the break between the inhibited and uninhibited period on the oxidation curves is sharper

for more effective antioxidants. Figure 7.2.2 shows an example for ethoxyquin and 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline inhibited oxidation, and the break is very sharp for both compounds.

Figure 7.2.2 Initiated and inhibited oxidation of methyl linoleate in chlorobenzene (6.8 x 10^{-1} M) at 50 °C. AIBN (4.5 x 10^{-2} M).



2,6-Dihydro-2,2,4-trimethyl-6-quinolone did not show any obvious inhibition period, but it did decrease the rate of oxygen uptake and the chain length.

Table 7.2.4 Initiated oxidation of methyl linoleate in chlorobenzene (6.8×10^{-1} M) containing 2,6-dihydro-2,2,4-trimethyl-6-quinolone at 50 °C. AIBN (4.5×10^{-2} M)

c ($M \times 10^4$)	ν	$\frac{d[O]}{dt}$ ($MM^{-1}s^{-1} \times 10^6$)
2.3	28.1	7.6
2.3	27.2	7.2
4.5	28.9	7.6
4.5	28.9	7.7
9.0	26.7	7.0
9.0	27.4	7.3
Average (s)	27.9 (0.9)	7.4 (0.3)

The chain length has decreased from 39.2 to 27.9, and the oxygen uptake from 10.3 (0.7) $\times 10^{-6}$ to 7.4 (0.3) $\times 10^{-6}$ $MM^{-1}s^{-1}$. At 30 °C the chain length decreased 184.6 (2.2) to 137.1 (30) and the rate from 2.3 (0) $\times 10^{-6}$ to 1.7 (0.4) $\times 10^{-6}$ $MM^{-1}s^{-1}$. This indicates a very slow reaction with peroxy-radicals, but also shows that the 6-quinolone does not owe all its antioxidant properties to reactions with alkyl-radicals. This agrees to results by Varlamov *et al* (111), where the quinone imine 4-(phenylimino)-cyclohexa-2,5-dien-1-one reacts not only with alkyl-radicals, but also with peroxy-radicals.

Table 7.2.5 shows the rate of oxygen uptake after the inhibition period of the antioxidants used and the results are illustrated in Figure 7.2.3.

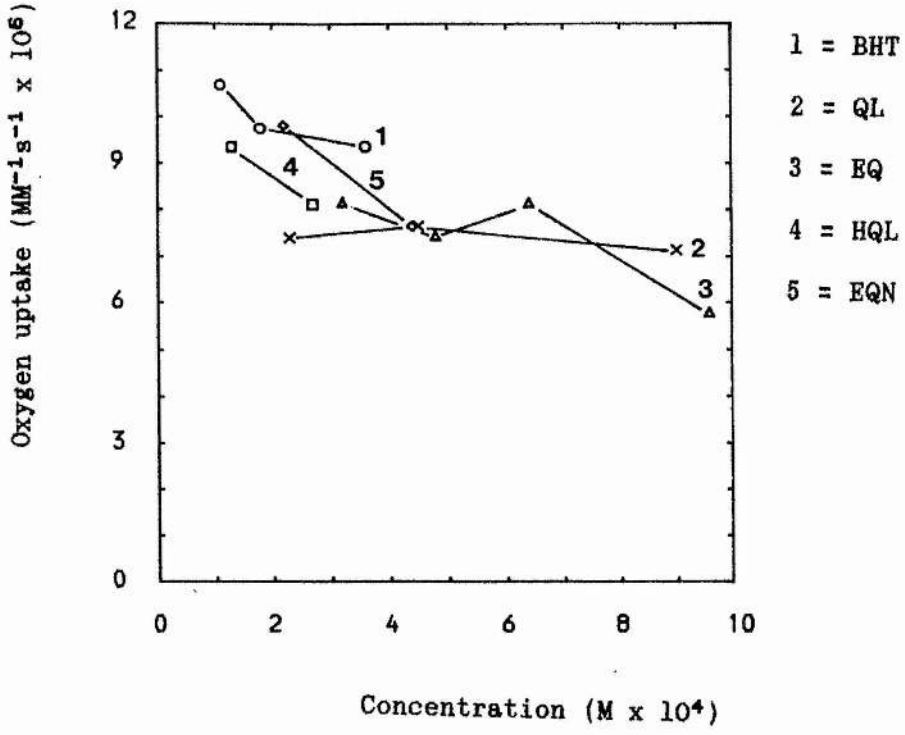
It is likely that formation of the 6-quinolone compound reduces the oxygen uptake after the inhibition period.

Table 7.2.5 Oxygen uptake after inhibition period of antioxidants in methyl linoleate in chlorobenzene (6.8×10^{-1} M) at 50 °C. AIBN (4.5×10^{-2} M).

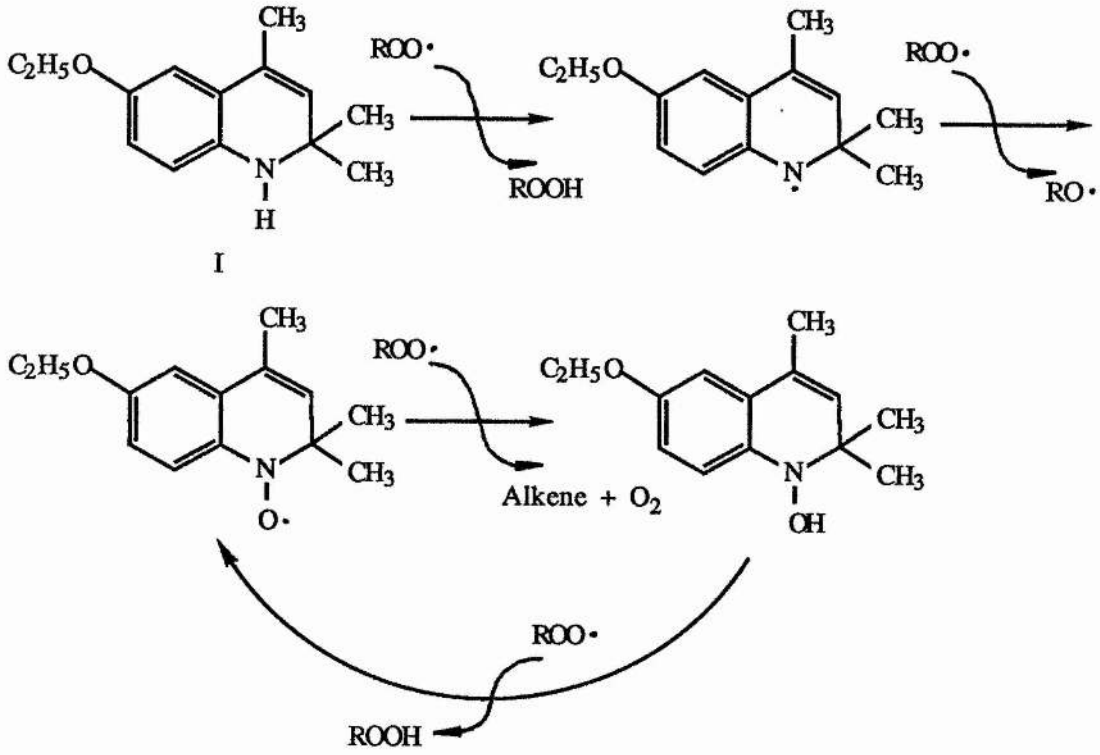
Antioxidant	c ($M \times 10^4$)	$\frac{d[O_2]}{dt}$[a] ($MM^{-1}s^{-1} \times 10^6$)
BHT	1.1	10.7 (0.6)
BHT	1.8	9.8 (0.8)
BHT	3.6	9.4 (0.8)
EQ	3.2	8.2 (0.1)
EQ	4.8	7.5 (0.5)
EQ	6.4	8.2 (0.2)
EQ	9.6	5.8 (0.4)
EQN	2.2	8.7 (1.6)
EQN	4.4	7.7 (0.8)
HQL	1.3	9.4 (0.4)
HQL	2.7	8.1 (0.1)

[a] Average of two

Figure 7.2.3 Oxygen uptake after inhibition period of antioxidants in methyl linoleate in chlorobenzene (6.8 x 10^{-1} M) at 50 °C. AIBN (4.5 x 10^{-2} M).



Ethoxyquin, in this system, can probably form its nitroxide which is itself a strong antioxidant. A possible reaction mechanism could be:



However due to various side reactions eg dimerisation or formation of the 6-quinolone the stoichiometric factor is only 2.3 - 2.5 instead of 3 - 4 or higher.

The high stoichiometric factor for the 6-hydroxyquinoline is probably due primarily to a release of two hydrogens. A nitroxide could also be formed or a radical addition could increase the factor from 2 to 2.5.

CHAPTER 8

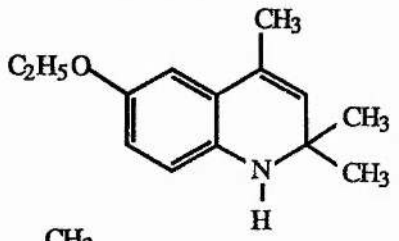
CONCLUSIONS AND DISCUSSION

Ethoxyquin (I) is a very powerful antioxidant in fish oils and fish meal. The mechanism of its antioxidant activity is complicated by the fact that it is itself oxidised to at least three products, two of which are themselves strong antioxidants.

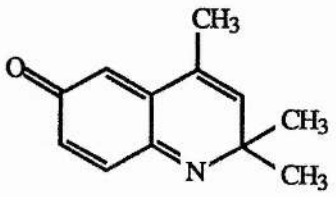
2,6-Dihydro-2,2,4-trimethyl-6-quinolone (II) was isolated and identified as an oxidation product of ethoxyquin in autoxidising methyl linoleate, crude fish oil and fish meal, but also as a product of tert-butoxy radical oxidation of ethoxyquin.

The 1,8' dimer (III) of ethoxyquin was identified in the same systems as was the 6-quinolone. However, it had no antioxidant properties.

The formation of ethoxyquin nitroxide (IV) was shown by ESR investigations, where ethoxyquin was converted to the amino-radical (V) which by reaction with peroxy-radicals can form the nitroxide. Ethoxyquin nitroxide was later made from a reaction of ethoxyquin and hydrogen peroxide in the presence of sodium tungstate, and isolated in pure form by preparative HPLC.



I



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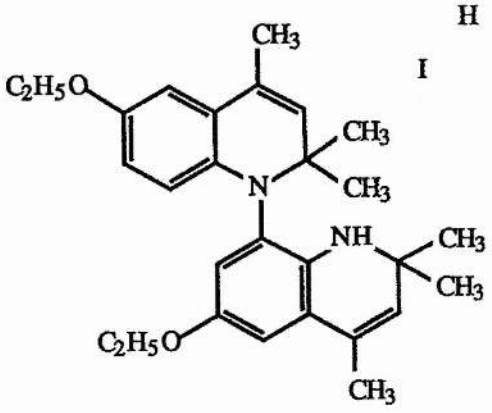
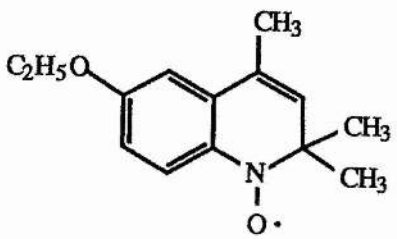
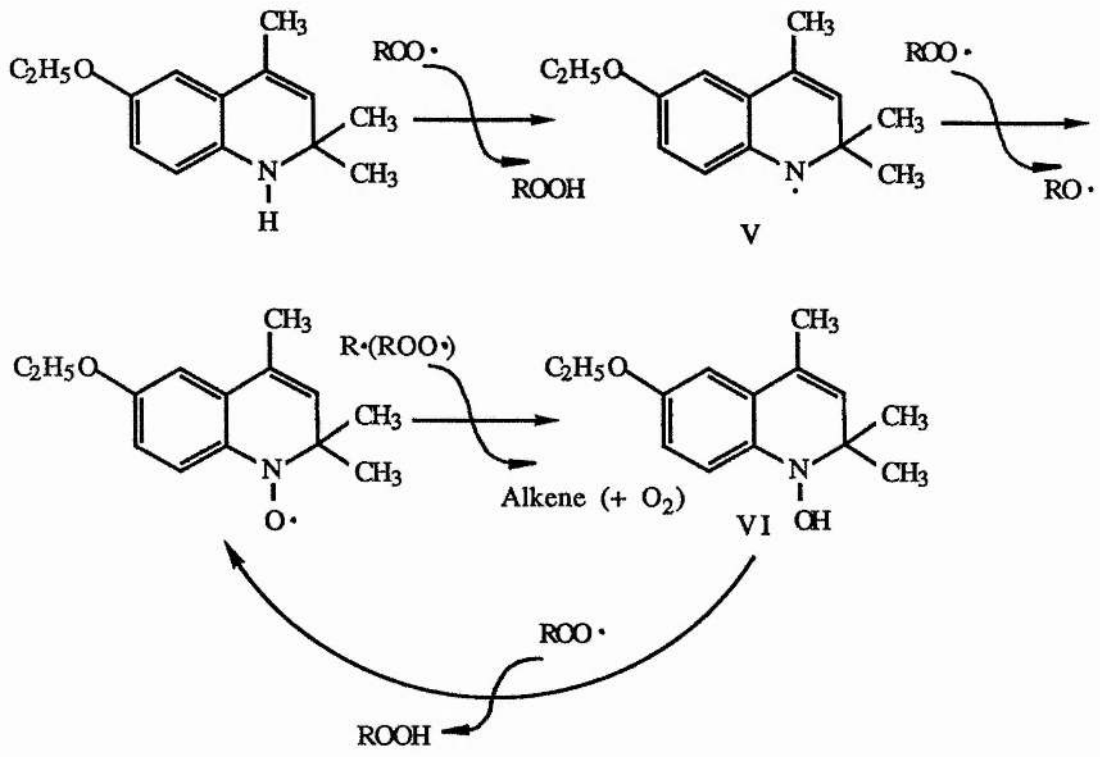


III

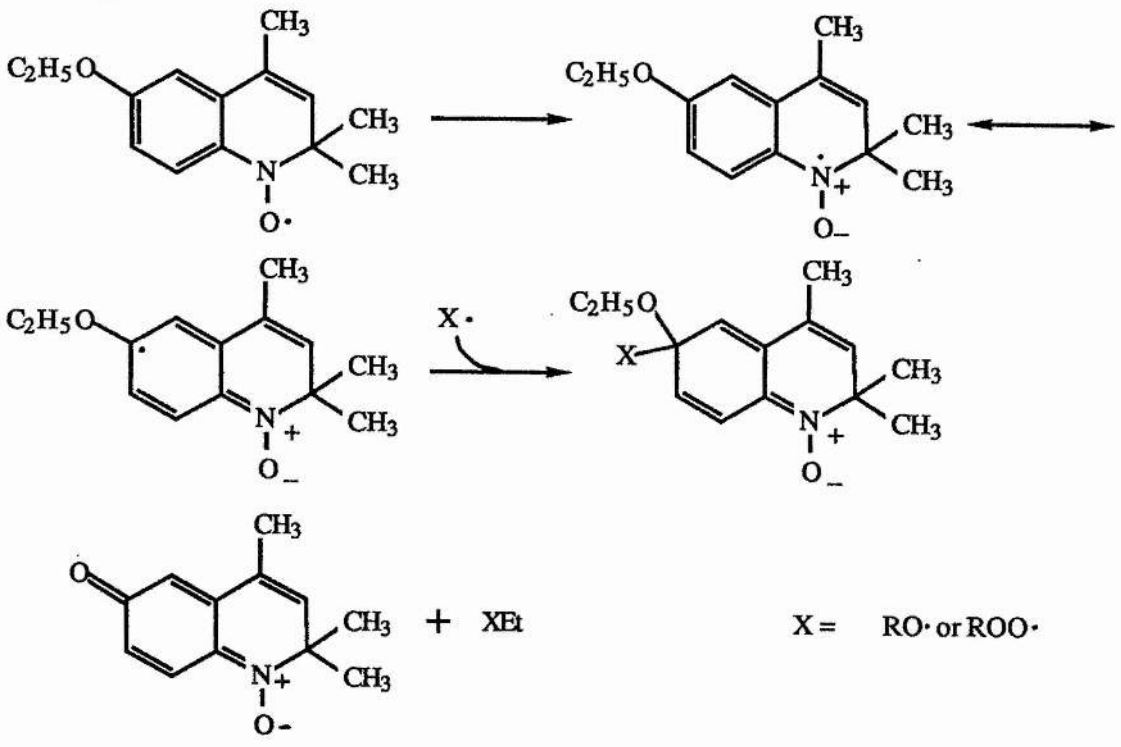


IV

The formation of ethoxyquin nitroxide and its antioxidant mechanism is formulated in the following sequence.

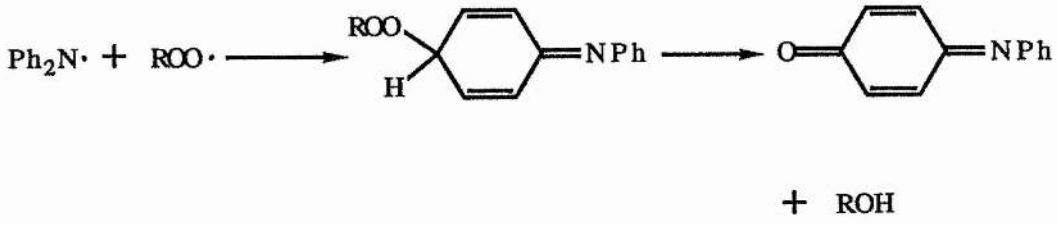


According to this scheme, the nitroxide abstracts a hydrogen from an alkyl- or a peroxy-radical to form hydroxylamine (VI) and an alkene. This proposal resembles the mechanism shown for the formation and antioxidant mechanism of nitroxides by Denisov *et al* (23) and Scott (10). However, the lifetime of ethoxyquin nitroxide is shortened due to side reactions, and the main product is 2,6-dihydro-2,2,4-trimethyl-6-quinolone-N-oxide (VII). This quinolone-N-oxide may be formed by a radical addition of position 6, followed by an elimination reaction to form the quinolone-N-oxide.



VII

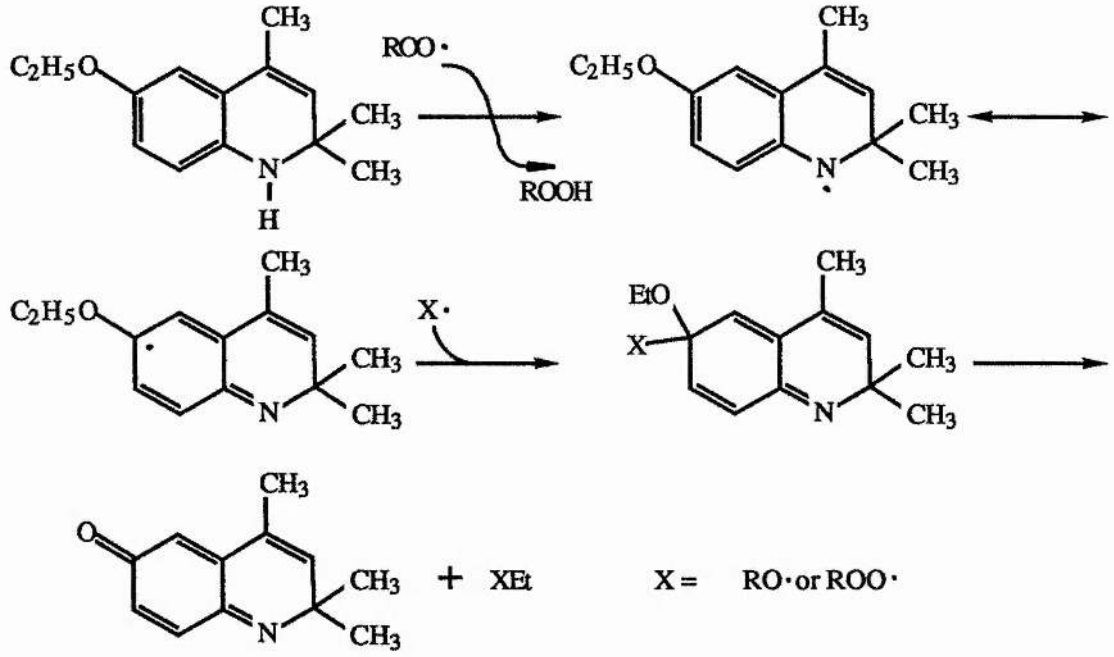
This is similar to the addition of a peroxy-radical in position 4 of the diphenylamine-radical (24), but here the keto-oxygen is provided by the peroxy-radical.



However, we have shown that the N-oxide is readily produced during preparation of the nitroxide (IV) in the absence of peroxy, alkoxy- or alkyl-radicals. The ease of this side reaction can explain why ethoxyquin nitroxide is not superior to ethoxyquin as an antioxidant.

The N-oxide (VII), when added to autoxidising oil, produced the corresponding quinolone (II), which also had strong antioxidant properties in oils and fish meal.

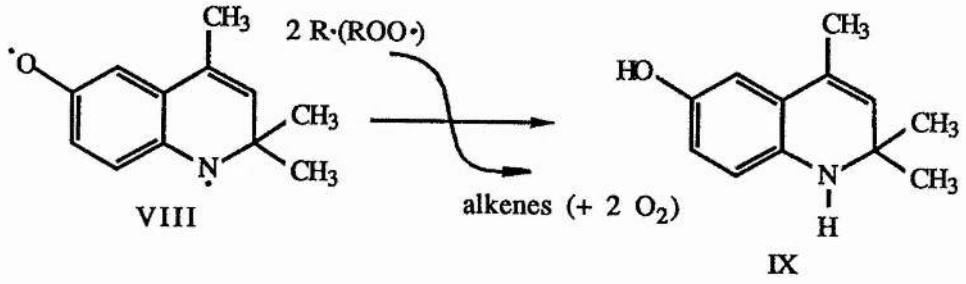
The formation of 2,6-dihydro-2,2,4-trimethyl-6-quinolone (II) from ethoxyquin may proceed by a similar sequence to the formation of the N-oxide (VII) from ethoxyquin nitroxide.



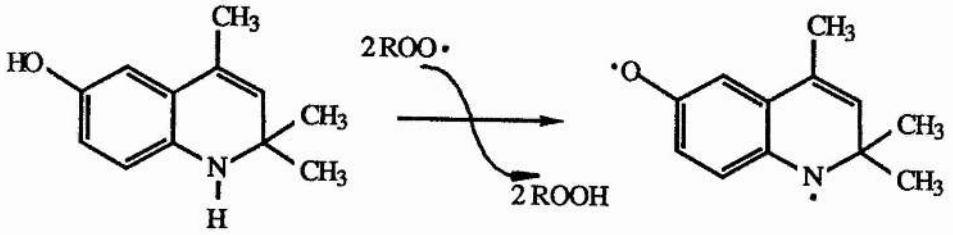
The stoichiometric factor for ethoxyquin of 2.3 - 2.5 can then be explained in part by the above mechanism, and partly by formation of the nitroxide (IV).

Although 2,6-dihydro-2,2,4-trimethyl-6-quinolone was found to be ineffective in AIBN-initiated linoleate autoxidation, it proved to be a very powerful antioxidant in fish oil and fish meal. In fact, whereas ethoxyquin sometimes showed initial pro-oxidant effects or a delayed inhibition, the quinolone was immediately effective. Its antioxidant properties can be linked to the fact that under certain conditions such as UV-light and in the presence of tert-butylhydroperoxide, it produces an ESR-spectrum, possibly of the di-radical (VIII).

This radical can then possibly undergo reduction by an alkyl- or a peroxy-radical or a straight radical addition can occur.



1,2-Dihydro-6-hydroxy-2,2,4-trimethylquinoline (IX) was prepared from ethoxyquin and found to be a very good antioxidant (n = 2.5). A stoichiometric factor of 2 is easily explained by the reaction;

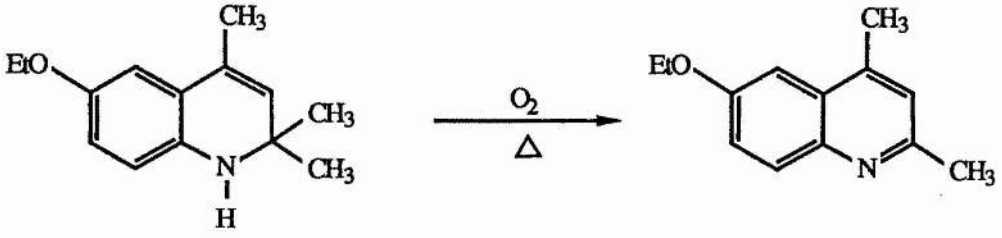


and the difference (n = 0.5) by a mechanism such as shown above for the 6-quinolone.

The 1,8'-dimer of ethoxyquin (III) was found to have no antioxidant properties. Its formation serves as an alternative to the formation of the 6-quinolone or ethoxyquin nitroxide, with consequent loss of antioxidant activity. Dimer formation probably becomes more significant at higher ethoxyquin concentration such as are sometimes used in fish meal.

2,4-Dimethyl-6-ethoxyquinoline (X) which can also be obtained

from ethoxyquin was shown to have pro-oxidant effects in fish oil. However, this is mainly a product of a high temperature reaction and is therefore hardly important in oils or fish meal under normal conditions.



X

CHAPTER 1 - GENERAL METHODS

Analytical high performance liquid chromatography (HPLC) was carried out on a silica column (25 cm x 4.6 mm id, 8 μ general purpose silica, Phase Sep.). The silica (3.6 g) was slurried in methanol (33 ml) and the column was packed (7000 psi) with methanol and then ethyl acetate. The solvent pump (Waters 6000) was coupled to an injector (Rheodyne 7125) with a 100 μl loop. A variable wavelength UV-detector (Waters 450) was used and a Spectra Physics (SP4100) computing integrator was used to print traces. For some experiments a Varian 9060 polychrom detector with a HP Think Jet printer was used. This unit records the UV-spectrum of each peak and calculates a so called purity parameter derived from the spectrum. Each compound has its own purity parameter which can be used to identify the compound, or monitor the homogenety of the peak (1).

Two preparative silica HPLC columns were packed for isolation and purification of the various compounds made. The silica (57 g, Li Croprep Si 60, 5-20 μ, Merck) was slurried in methanol (150 ml) and the column (25 cm x 22 mm id) was packed (4000 psi) with methanol (500 ml) followed by ethyl acetate (500 ml). A smaller preparative column (25 cm x 10 mm id) was packed from the silica used for the analytical column. The silica (16 g) was slurried in methanol (150 ml) and the column was packed with methanol (300 ml) followed by ethyl acetate (200 ml).

The mobile phase was isopropanol in hexane (HPLC grade) and a mixture such as 2% ($^{V}/_{V}$) isopropanol in hexane is referred to as 2% IPO.

Gas chromatographic (GC) analyses of ethoxyquin and 2,4-dimethyl-6-ethoxyquinoline were carried out on a PYE 104 chromatograph with a flame ionisation detector. The packed glass column (180 cm x 2 mm id) contained SE-30 on Chrom WHP 80/120 Mesh. The gas flow rates and oven temperature conditions are described for the individual experiments in later chapters.

For methyl ester analysis, an HP 5880 system operated in the splitless mode was used. The capillary column was a Durabond (0.25 μ film, 25 m x 0.2 mm id, J & W Scientific Inc., California, USA). The carrier gas was nitrogen at 17 psi pressure and hydrogen and air were at 40 and 20 psi respectively. The initial oven temperature (50^{o}C) was held for 3 minutes and then raised at 25 ^{o}C/min to 140 ^{o}C and there after at 3 ^{o}C/min to 240 ^{o}C. The injector and detector temperature were maintained at 300 ^{o}C and the total run time was 55 minutes.

The GC-MS system used was an HP5790 chromatograph linked to a VG 16F mass spectrometer in EI-mode (70 eV) with the inlet and source temperatures at 250 and 200 ^{o}C respectively. VG 2250 DS was used for data processing. The fused silica capillary column was a nonpolar bonded methyl silicone (25 m x 0.2 mm id, HP-ultra performance) and the carrier gas was helium (10 psi). The initial oven temperature of 50 ^{o}C was held for 3 min and then raised at 10 ^{o}C/min to a final temperature of 260 ^{o}C held for 10 minutes. The injector temperature was 260 ^{o}C.

Thin layer chromatography (TLC) was done on silica plates from Merck and Whatman (silica on glass) and from Camlab (silica on aluminium). Plates were also prepared by spreading aqueous silica slurry (2g water/g silica) over glass plates. All plates were 20 x 5

cm in area and 0.25 mm thick. The developing solvent was usually a mixture of petroleum ether (bp 40-60 °C) and diethyl ether. Solvent mixtures such as 20% ($^{V}/_{V}$) diethyl ether in petroleum ether will be referred to as PE 20. The chromatograms were viewed under UV-light or by spraying with an ethanolic solution (10% $^{W}/_{V}$) of phosphomolybdic acid, followed by heating over 120 °C for 1-2 minutes.

Liquid scintillation counting was done on a Isocap/300 counter, model 6868. All samples were counted in low-potassium scintillation tubes and were dissolved in NE 260 liquid scintillation solution (Nuclear Enterprises, Edinburgh). The counter was used in program 2 mode and counting time was 20 minutes per sample. Efficiency standard curves were prepared by the use of quenching standards (Amersham - Searle).

^{14}C-scanning of TLC plates was done on a Panax radio thin layer scanner with the slit width set at 2 mm. The counting gas was butane (1.5%) in helium.

Proton nuclear magnetic resonance (NMR) spectra were recorded on a Bruker WP-80 instrument at 80 MHz. Samples were dissolved in deuteriated chloroform or acetone (0.5 M). ^{13}C-NMR spectra were recorded on a Varian CFT-20 instrument at 20 MHz using solutions in deuterochloroform (0.5 - 1.0 M).

Mass spectra were recorded on an AEI MS902 double focusing mass spectrometer. The electron source was set at 70 eV and the source heater at 250 °C.

UV-spectra were recorded on a Pye-Unicam SP8-100 instrument and IR-spectra on a Perkin Elmer 1420 ratio recorder, using thin films between sodium chloride discs. Microanalysis were done on a

Carlo Elba 1106 elemental analyser.

CHAPTER 2 - PREPARATION OF ETHOXYQUIN AND SOME OF ITS DERIVATIVES

2.1.1 Preparation of unlabelled and labelled ethoxyquin

Redistilled acetone and p-phenetidine (Aldrich 98%, used without further purification) in the quantitites indicated below, were refluxed with iodine (1% of p-phenetidine weight) at 145-150 °C in xylene (50 ml). All the reactions were carried out under nitrogen in the dark and in the second reaction a "Dean and Stark" receiver was introduced between the flask and the condenser in order to estimate the water formed.

	p-phenetidine (mole/g)	acetone (mole/g)
1	0.15/20.6	0.075/4.3
2	0.1 /13.7	0.1 /5.8
3	0.1 /13.7	0.2 /11.6

Samples (0.1-0.2 g) were taken from the reaction mixtures at different times for GC-examination (SE-30 column). The initial oven temperature of 100 °C was raised at 4 °C/min up to the final temperature 200 °C. The carrier gas was nitrogen at 33 ml/min. The samples were dissolved in a solution (10 ml) of N,N-dimethyl-aniline in diethylether (1.33 g/l and later 1.07 g/l). The N,N-dimethylaniline served as an internal standard in order to allow estimation of ethoxyquin formed. The samples were also examined by TLC (PE50).

One reaction was carried out without xylene. Acetone (23.2 g) and p-phenetidine (27.4 g) were heated under reflux with iodine (0.34

g) and the formation of ethoxyquin was monitored as before.

2.1.2 Preparation of unlabelled ethoxyquin

Following these preliminary experiments, p-phenetidine (68.6 g), acetone (29.1 g) and iodine (0.85 g) were refluxed in xylene (250 ml) for 28 hours under nitrogen in the dark.

The reaction solution was extracted with aqueous hydrochloric acid (0.1 M, 300 ml and 1.0 M, 3 x 500 ml). Each extract was separately made basic with sodium hydroxide and extracted with diethyl ether (3 x 250 ml) which was then dried over sodium sulphate, filtered and evaporated to dryness on a rotary evaporator. It was apparent from TLC (PE60) that the last two acidic extracts contained the majority of ethoxyquin formed. These were combined and purified by silica adsorption column chromatography (70 cm x 28 mm id).

The silica column was eluted with portions of petroleum ether (500 ml) containing increasing concentrations of diethyl ether (P, PE5, PE10 etc) and the fractions were examined by TLC (PE60).

2.1.3 Preparation of ^{14}C-ethoxyquin

2,4-^{14}C-Ethoxyquin was made by modification of the method of Müller (2) using 2-^{14}C-acetone (500 μCi, 57.2 μCi/mmole, Amersham International, UK) obtained in sealed quickfit glass tubes.

The first preparation involved refluxing a mixture of acetone containing the radiolabelled material (1.0 g), p-phenetidine (2.4 g) and iodine (3.9 x 10^{-2} g) in redistilled xylene (10 ml) for 28 hours under the same conditions as before. After drying over sodium sulphate, the product was purified by adsorption column chromatography (40

cm x 22 mm id) on silica. The column was eluted with portions of petroleum ether (100 ml) containing diethyl ether (P, PE5, PE10 etc) and fractions (25 ml) were collected. The initial and final reaction mixture were examined by TLC (PE60) and a radio thin layer scanner. The radioactivity was measured in a portion (0.6 ml) of each column fraction.

In a later preparation, redistilled acetone containing the radiolabelled material (1.0 g), redistilled p-phenetidine (1.2 g) and iodine (1.5 x 10^{-2} g) were refluxed in xylene (10 ml) under the same conditions as before. After 32 hours the solution was cooled, aqueous sodium hydroxide (2 M, 25 ml) was added and the mixture was extracted with hexane (3 x 25 ml). The combined extracts were washed with aqueous sodium hydroxide (1 M, 25 ml) and dried over sodium sulphate before removing the hexane and the xylene, first on a rotary evaporator and finally on a vacuum pump.

The product (dark oil, 1.53 g) was divided into four equal portions and purified on preparative HPLC (25 cm x 22 mm id, 2% IPO at 4 ml/min). Fractions were collected (initially 4 ml) and examined by analytical HPLC (2% IPO at 2 ml/min). The radioactivity was determined on a portion (0.1 ml) of each fraction from the second preparative run. A sample of the non-purified oil was also examined by analytical HPLC and fractions collected for radioactivity measurements.

Fractions from the preparative runs, containing more than 99% ethoxyquin by HPLC, were combined and made up to volume (350 ml) in hexane. The radioactivity was measured in two samples (0.05 ml) and a further sample was examined by analytical HPLC (2% IPO at 2 ml/min and methanol at 10 min) and fractions were collected for

radioactivity counting as before.

The whole volume of each analytical HPLC fraction was used for radioactivity measurements, except for the last methanol fraction where a portion (3 ml) was taken to dryness before counting.

2.2 Preparation of 2,4-dimethyl-6-ethoxyquinoline

Ethoxyquin (150 mg), purified by silica adsorbtion column chromatography, was heated (250 °C) in five equal portions in open flasks. At two minute intervals one sample was dissolved in diethyl ether and examined by TLC (PE50) and GC (SE-30). The oven temperature was isothermal at 160 °C and the carrier gas was nitrogen at 15 psi (flow rate unknown).

The quinoline derivative was prepared from ethoxyquin in two ways. In the first method, commercial ethoxyquin (Rexoquin 100, Rexolin, 2.0 g) was heated (280 °C, 13 min) in an open flask. The desired product was then isolated by silica adsorption column chromatography (70 cm x 22 mm id). The column was eluted with portions of petroleum ether (600 ml) containing diethyl ether (P, PE5, PE10 etc).

The second preparation was a modification of a method by Gallagher and Stahr (3). Commercial ethoxyquin (Rexoquin 100, 7.1 g) in xylene (100 ml) was refluxed for 18 hours while bubbling oxygen through. The resulting mixture was shaken with chloroform (100 ml) and aqueous hydrochloric acid (1 M, 200 ml), and the aqueous phase removed and made basic with sodium hydroxide. This was extracted with chloroform (2 x 100 ml) and the chloroform was washed with water (2 x 100 ml) before drying over sodium sulphate, filtering and removing the solvent on a rotary evaporator. The product (mp 86-87

°C) was purified by crystallisation from petroleum ether and examined and found to be pure by TLC.

2.3 Preparation of 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline-nitroxide

A modification of method by Lin and Olcott (4) was used to make ethoxyquin into its nitroxide. Ethoxyquin (1.0 g, purified by silica adsorption column chromatography) was dissolved in ethanol (40 ml) and added to a solution of sodium tungstate (102 mg, Koch-Light Lab) and EDTA disodium salt (177 mg) in water (8 ml). After adding hydrogen peroxide (30%, 10 ml), the solution was stirred at room temperature for 4 hours. Water (60 ml) was then added and the solution saturated with potassium bicarbonate and extracted with benzene (2 x 60 ml). The benzene extract was washed with water (4 x 100 ml), dried over potassium bicarbonate and filtered. The solvent was removed on a rotary evaporator and a vacuum pump.

Isolation of the nitroxide from the viscous red oily product by adsorption column chromatography (silica and aluminium oxide pH 9.3 - 9.7, chloroform) was unsuccessful, but purification was later achieved by preparative HPLC.

The crude product (400 mg) was dissolved in hexane (2 ml) and injected onto a preparative HPLC column (25 cm x 22 mm id, 7% IPO at 7 ml/min). The crude product and fractions from the preparative HPLC were examined by analytical HPLC (2% IPO at 2 ml/min for 5 min, 3 ml/min for 3 min and finally 4 ml/min).

2.4 Preparation of 2,6-dihydro-2,2,4-trimethyl-6-quinolone

Ethoxyquin (0.49 g, purified by preparative HPLC) was dissolved in ethanol (50 ml). An equimolar amount of tert-butylhydroperoxide (70%, 0.31 ml) was added, followed by an aqueous solution of ammonium ferrous sulphate (0.88 g in 20 ml). After stirring at room temperature for 26 hours, aqueous hydrochloric acid was added (1 M, 100 ml) and the mixture was extracted with hexane (3 x 100 ml). The hexane was extracted with aqueous hydrochloric acid solution (1 M, 2 x 100 ml) and all the acid phases were combined and made basic with sodium hydroxide. They were then extracted with hexane (4 x 100 ml) and the hexane extract was washed with aqueous sodium hydroxide (1 M, 3 x 100 ml). After drying over sodium sulphate and removing the solvent on a rotary evaporator and a vacuum pump, a red oil (0.24 g) was obtained.

During the reaction, the mixture was examined by TLC (PE60) and the crude product by analytical HPLC (2% IPO at 2 ml/min).

The desired product was purified by preparative HPLC (25 cm x 22 mm id, 4% IPO at 6 ml/min), and when needed, a further purification was provided by a smaller preparative HPLC column (25 cm x 10 mm id, 3% IPO at 3 ml/min). All fractions were examined by analytical HPLC (2% IPO at 2 ml/min).

2.5 Preparation of the 1,8'-dimer of ethoxyquin

From the reaction described in section 2.4, the hexane extract of the acidified reaction mixture was dried over sodium sulphate and the solvent was then removed on a rotary evaporator to leave a solid material (0.20 g). The product was purified by preparative HPLC (25 cm x 22 mm id, 2% IPO at 3 ml/min) and fractions were examined by

analytical HPLC (2% IPO at 2 ml/min).

2.6 Preparation of 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline

Ethoxyquin (5 g, purified by silica adsorpiton column chromatography) was refluxed (120 °C) with hydrobromic acid (48%, 20 ml). The flask was opened every 30 minutes to release the ethyl bromide formed. After three hours, the flask was left open for an hour while boiling away most of the liquid. After drying on a rotary evaporator, water (50 ml) was added followed by aqueous sodium hydroxide (2 M) until pH 10 was reached. The solution was then filtered, and the solids were washed with water (100 ml). They were then mixed with benzene (200 ml) and the water/benzene azeotrope was removed on a rotary evaporator to give a green powder (3.65 g). By repeated crystallisation from toluene, colourless crystals were obtained (mp 170-172 °C (decomp.)). The product was examined and found to be pure by TLC (PE 60).

CHAPTER 3 - ESR STUDIES OF ETHOXYQUIN AND SOME OF ITS OXIDATION PRODUCTS

ESR spectra (3335 Gs, 9.3 GHz) were recorded with a Bruker ER200D spectrometer.

Ethoxyquin was purified by either preparative HPLC or silica adsorption column chromatography. The spectra were obtained from solutions in n-heptane, toluene or di- tert-butylperoxide (1×10^{-2} M) and one solution (Figure 3.2.3) was degassed by repeated freeze-thaw cycles before recording of the spectrum.

Ethoxyquin nitroxide, purified by preparative HPLC, was dissolved in benzene (1×10^{-4} M), and nitrogen was passed through the solution before recording of the spectra.

Solutions of 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline and 2,6-dihydro-2,2,4-trimethyl-6-quinolone in tert-butylbenzene (1×10^{-2} M) were degassed by repeated freeze-thaw cycles before recording of the spectra.

Computer simulated spectra were done by use of OCPE program (Univ of Indiana, 1972, No 209, J. Heinzer). The simulation in Figure 3.2.3 was obtained with the help of R.A. Jackson (Univ of Sussex, programs Correlation Match Seek).

CHAPTER 4 - LIPID ANALYSIS

4.1 Oil extraction from herring and mackerel

The frozen fish was thawed overnight and the head and spine removed the next day. The residue was minced and stirred with sodium sulphate (30 g in 100 g mince). The dried mince was then centrifuged (200 ml flasks, 2000 rpm, 20 min) and the upper layer of oil removed with a pipette.

4.2 Oil content of fish meal

The oil content of the fish meal was determined by a modification of the Bligh and Dyer procedure (5). Meal (5 g) was weighed in a centrifuge bottle (200 ml) and chloroform (20 ml), methanol (40 ml) and water (16 ml) added. The mixture was homogenised with an Ultraturrex for 3 minutes in an icebath. Chloroform (20 ml) was added and the mixture homogenised for one minute. Finally, water (20 ml) was added and the mixture was homogenised for a further one minute. The sample was then centrifuged (2000 rpm, 0 ^{o}C, 20 min) and the methanol/water phase removed. A portion (10 ml) of the chloroform phase was measured with a pipette into a preweighed flask (100 ml) and the chloroform was removed on a rotary evaporator and finally on a vacuum pump.

4.3 Fatty acid analysis

The fatty acid composition of the oils and the chloroform extractables from the fish meals were determined by a slight modification of the method used at the Torry Research Station. All samples were kept under nitrogen (-20 ^{o}C) and were analysed at the

same time.

The oil (40-50 mg) was weighed into a flask (50 ml) and a benzene solution (2 ml) of tricosanoic acid (73.4 mg) and BHT (28.9 mg) was added. This was followed by methanoloic sodium hydroxide (0.5 M, 7.5 ml). The tricosanoic acid serves as an internal standard to give an estimate of the weight of each fatty acid. The BHT is an antioxidant and inhibits oxidation during sample preparation. After refluxing for 30 minutes, boron trifluoride in methanol (14%, 7.5 ml) was added and the solution was refluxed for a further 30 minutes. The first reflux period releases the fatty acids by saponification, and the second step makes methyl esters under acidic conditions.

The solution was then cooled, distilled water (20 ml) was added and the mixture extracted with hexane (2 x 30 ml). The hexane extracts were washed with distilled water (2 x 15 ml) and dried with anhydrous sodium sulphate. After diluting with hexane (1:6), the solution was put into a crimped vial with a rubber seal.

Chromatographic analyses were carried out on a Hewlett Packard 5880 GC, operated in the splitless mode. The carrier gas was nitrogen at 17 psi pressure, and hydrogen and air were at pressures of 40 and 20 psi respectively. The column was capillary polar phase Durabond (J & W Scientific Inc., California, USA, 0.25 μ film, 25 m x 0.2 mm id). The initial oven temperature (50 ^{o}C) was held for 3 minutes, then raised to 140 ^{o}C at 25 ^{o}C/min and finally to 240 ^{o}C at 3 ^{o}C/min. The injector and detector were maintained at 300 ^{o}C.

All samples were analysed in duplicate with two injections from each vial. A fish oil of a known composition was used as a standard. Its methyl esters were injected between every four samples and a

response factor was calculated for each fatty acid by taking the ratio of the observed value to the known value. By multiplying this factor by the percentage of fatty acid in the sample, the actual composition is obtained.

CHAPTER 5 - AUTOXIDATION EXPERIMENTS

5.1 General

All oil autoxidation experiments were done at the same temperature (30 °C) on 5 g samples in conical flasks (25 ml), which were constantly shaken. The fish meal samples (40 - 50 g) were autoxidised at the same temperature as the oil, in stationary conical flasks (100 ml). After inserting the flasks in the water bath, the manometers were kept open for one hour to allow for thermal equilibration. After every reading, air exchange in the flasks was secured by sucking air out a few times and letting fresh air in.

To introduce the antioxidants into the oil, a solution (usually in hexane) was prepared and appropriate volume (1 - 5 ml) was measured into the flask with a pipette. The solvent was then evaporated under nitrogen and the oil added and mixed thoroughly. Two methods were generally used to mix the antioxidants in the fish meals. One was based on adding antioxidant to a small amount of oil (same producer as fish meal) by the method described earlier and then mixing that with the meal in a Kenwood kitchen stirrer. The other method was to measure a volume of the antioxidant solution with a pipette onto the meal and shake it with a large amount of hexane (200 ml). The hexane was then removed on a rotary evaporator until the sample reached its original weight. The controls were treated the same way as the other samples.

Unless otherwise stated, the samples were autoxidised in duplicate and the results shown as an average oxygen uptake per gram of oil (estimated 10% for fish meal).

5.2 Ethoxyquin in fish oils

The effects of ethoxyquin on the autoxidation of various fish oils was studied, and in one experiment compared to BHT. In another experiment the effects of oxidation level of the oil on ethoxyquin antioxidant activity was studied. Ethoxyquin was added to mackerel oil at different levels of oxygen uptake (0, 20, 50 and 90 μmole/g). The ethoxyquin was mixed in a small sample (0.5 g) of the original oil and then added to the autoxidised oil such that the final concentration came to 0.1% and then the sample was autoxidised further.

Results illustrated in Figures 5.2.1, 5.2.2 and 5.2.5 are for single samples, but the controls were in duplicate. Results in Table 5.2.1 are combined from two experiements.

5.3 Ethoxyquin in fish meal

In experiments described in Figures 5.3.1 and 5.3.2 the ethoxyquin was added to the meal by the previously described oil method. BHT was also added by this method.

The solvent method of mixing the antioxidants to the meal was described earlier (section 5.1). The so called vacuum method involved adding the antioxidant in a small amount of solvent onto the meal. The meal was then heated under vacuum (90 ^{o}C, 10-15 mm Hg) while being rotated.

Throughout this study a single sample was autoxidised for each antioxidant concentration, but the controls were in duplicate.

5.4 Ethoxyquin nitroxide in oils

Ethoxyquin nitroxide was added to the oil as has been described for ethoxyquin. A single sample was used for each anti-oxidant concentration.

5.5 2,6-Dihydro-2,2,4-trimethyl-6-quinolone and 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline in oils and fish meal

Methyl linoleate (4 x 5 g) was autoxidised for 4 days (30 °C) before adding ethoxyquin (5%) and further autoxidising the mixtures for 4 days. After dissolving the samples in hexane (50 ml) and extracting with aqueous hydrochloric acid (4 x 40 ml, 2 M) the acid phase was washed with hexane (2 x 75 ml) and then made alkaline with sodium hydroxide. The alkaline solution was extracted with hexane (4 x 100 ml) and the hexane extracts washed with water (100 ml), dried over sodium sulphate, filtered and the solvent was removed by a rotary evaporator to give a yellow oil (0.78 g). This was fractionated by preparative HPLC (25 cm x 22 mm id, 4% IPO at 6 ml/min) and a fraction collected between 198 and 228 ml gave a yellow solid (1% yield) which was shown to be 2,6-dihydro-2,2,4-trimethyl-6-quinolone. This compound was also made by oxidation of ethoxyquin with tert-butylhydroperoxide.

Single samples of fish meal containing the antioxidant were autoxidised.

1,2-Dihydro-6-hydroxy-2,2,4-trimethylquinoline was only tested in mackerel oil.

5.6 1,8'-Ethoxyquin dimer and 2,4-dimethyl-6-ethoxyquinoline in oils

Samples of mackerel oil containing the 1,8'-dimer of ethoxyquin were autoxidised as has been described earlier. The effects of 2,4-dimethyl-6-ethoxyquinoline on autoxidation of oil were examined in cod liver oil.

CHAPTER 6 - OXIDATION REACTIONS OF ETHOXYQUIN AND SOME OF ITS OXIDATION PRODUCTS

6.1 Ethoxyquin reactions

An equimolar solution of ethoxyquin and tert-butylhydroperoxide (7.4×10^{-5} M) in ethanol was kept in the dark at room temperature and the UV-spectrum (ethoxyquin) was examined over a period of 24 hours.

Methyl linoleate (2 x 5 g) was autoxidised for 120 hours (30 °C) and ethoxyquin, in fresh methyl linoleate (10.2% in 0.55 g), was then added such that the final concentration of ethoxyquin was 1%. Samples were examined by TLC (PE60) for the formation of new products. In a similar experiment (ethoxyquin in methyl linoleate), the sample was dissolved in hexane and extracted with acid and the reaction product isolated by HPLC (section 5.5).

South African fish meal was extracted by a modified method of Bligh & Dyer (5) (section 4.2) after having been autoxidised (30 °C) for 60 days in the presence of ethoxyquin (0.5%). Chloroform soluble material (0.5 g) was separated by preparative HPLC (25 cm x 22 mm id, 2% IPO at 5 ml/min) and the fractions were analysed by analytical HPLC (25 cm x 4.6 mm id, 0.5% IPO at 1 ml/min, 229 nm).

South African meal was autoxidised for one week (30 °C) prior to addition of ethoxyquin (0.5%). After autoxidation for a further one week the chloroform soluble material was extracted with acid as in the previous experiment. The relative amounts of ethoxyquin and 2,6-dihydro-2,2,4-trimethyl-6-quinolone were then estimated by analytical HPLC (25 cm x 4.6 mm id, 2% IPO at 2ml/min). The relative response at 254 nm was estimated by using solutions of the pure

compounds.

A solution of 2,4-^{14}C-ethoxyquin (0.308 g) in hexane (500 ml) was shaken with South African meal (300 g) for 1 minute. The hexane was then removed on a rotary evaporator and the sample kept at 25-30 ^{o}C in an open beaker. Samples (2 x 5 g) were taken over a period of 9 weeks, homogenised with a mixture of methanol, chloroform and 1.0 M aqueous hydrochloric acid (50/20/15 ml, 3 min) and centrifuged (20 min). The residual solids were retreated twice (homogenised for 1 minute) and the combined solutions made up to volume in methanol (250 ml, Total extract). Samples (1 ml) were taken, the solvent removed and the radioactivity was measured. Most of the solvent from the remaining solution was then removed on the rotary evaporator. The residue was mixed with hexane (50 ml) and extracted with aqueous hydrochloric acid (1 M, 25 ml) and the organic layer was treated twice more with hexane (50 ml) and hydrochloric acid (1 M, 25 ml). The hexane solution was finally washed with aqueous hydrochloric acid (1 M, 3 x 25 ml), dried over sodium sulphate and made up to volume (250 ml, Hexane 1).

The combined acid extracts were made alkaline with aqueous sodium hydroxide (2 M), extracted with hexane (3 x 100 ml) which then was washed with aqueous sodium hydroxide (1 M, 2 x 100 ml), dried over sodium sulphate and made up to volume (500 ml, Hexane 2). Radioactivity measurements were done on Hexane 1 (2 ml) and Hexane 2 (10 ml) after removing the solvent. The remains of Hexane 2 (480 ml) were then reduced to 1 ml and separated into fractions (2 ml) by preparative HPLC (25 cm x 10 mm id, 2% IPO at 2 ml/min) and a portion (0.5 ml) of each fraction was used for radioactivity measurements.

A portion of the meal after extraction was dried under vacuum and a small sample (0.1 g) was digested in aqueous sodium hydroxide (2 M, 10 ml, 80 °C for 24 hours). The radioactivity of a portion (1 ml) of the digest was then determined.

6.3 GC-MS experiments

South African oil was autoxidised (40 °C) for 2 days before adddition of ethoxyquin or some of its oxidation products (0.1%). After further autoxidation for 4 days, each sample was dissolved in hexane (150 ml) and extracted with aqueous hydrochloric acid (1 M, 2 x 100 ml) and the aqueous phase was then washed with hexane (2 x 75 ml). After making the acid phase alkaline with aqueous sodium hydroxide (1 M), it was extracted with hexane (2 x 100 ml). The hexane was washed with aqueous sodium hydroxide (1 M, 50 ml) before drying over sodium sulphate, and each sample was analysed by GC-MS.

CHAPTER 7 - INHIBITION OF AIBN INITIATED AUTOXIDATION OF METHYL LINOLEATE BY ETHOXYQUIN AND THREE OF ITS OXIDATION PRODUCTS

Methyl linoleate (Sigma, 99%) was found to be free of oxidation products by TLC. AIBN (2,2'-bis(isobutyronitrile), Fluka AG) was used as obtained and chlorobenzene was redistilled before use.

Ethoxyquin and related compounds were purified as described earlier (Chapter 2). BHT (BDH) was freed from oxidation products by passing it through a silica column with petroleum ether. It was then found to be pure by TLC.

For a typical oxidation measurement, a petroleum ether solution of the antioxidant (diethyl ether was required for 1,2-dihydro-6-hydroxy-2,2,4-trimethylquinoline) was measured into a calibrated flask (25 ml). After removing the solvent by blowing with nitrogen, methyl linoleate (0.25 g) was accurately weighed into the flask, AIBN in chlorobenzene (1 ml, 5.8 x 10^{-2} M) was added and mixing secured by gently rotating the flask.

After inserting the flasks, joined to the manometers, into the water bath, a period of 10-15 minutes was allowed for thermal equilibration. The results were then extrapolated to zero time. A flask containing only the AIBN solution served as a thermobarometer, and it also corrects for nitrogen evolved and oxygen absorbed by the initiator.

The manometric fluid was a solution of NaBr (44 g), bromothymol blue (0.3 g) and Micro (0.3 g, detergent) in water (1000 ml) to give a density 1.033 g/ml (10000 mm/atm).

To secure maximum radical efficiency all flasks were silanized

before each use so that "wall-effects" were minimised.

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